THE RNA-DEPENDENT RNA POLYMERASE OF POLIOVIRUS AND THE RNA AND DNA-DEPENDENT DNA POLYMERASES OF ROUS SARCOMA VIRUS

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Table of Contents

Preface i

Acknowledgments ii

Abbreviations iii

Abstract v

I. Part I - The RNA-Dependent RNA Polymerase of Poliovirus . 1

A. Introduction. 1

B. Materials and Methods 12

Cells and Virus. 12

Preparation of particulate poliovirus RNA polymerase 12

Conditions for assay of poliovirus RNA polymerase 13

Extraction of RNA 14

RNase digestion of purified RNA 14

Salt fractionation of purified RNA 15

Preparation of polyacrylamide gels 15

Electrophoresis in polyacrylamide gels 15

Protein determinations 16

Reagents 16

C. Results 18

Kinetics of appearance of poliovirus RNA polymerase 18

Electrophoretic analysis of the product of the viral RNA polymerase 18

Kinetics of enzymatic labeling of RI, RF and SS RNA 28

Effect of DOC on enzyme function 39

Effect of guanidine on the products of enzymatic synthesis 55

XUM

D. Discussion 62

II. Part II - The RNA and DNA-Dependent DNA Polymerases of Rous Sarcoma Virus. 69

A. Introduction 69

B. Materials and Methods. 73

Preparation of virus 73

Conditions for assay of the RSV DNA polymerase. 73

Conditions for assay of the DNA-dependent DNA polymerase. 74

Preparation of DNA. 74

Preparation of single-stranded DNA. 74

Extraction of nucleic acids 75

Reagents. 75

C. Results. 76

pH ionic and temperature requirements of virus-associated RNA dependent DNA polymerase 76

Delineation of two enzymatic activities 79

Stimulation of DNA synthesis by exogenous DNA 82

Association of DNA-dependent DNA polymerase with RSV virions 82

Template requirements of the DNA-dependent DNA polymerase. 87

Solubilization of the RSV DNA polymerase. . . . 90

Ammonium sulfate fractionation. 91

Determination of the sedimentation coefficient of the isolated DNA polymerase. 97

Extent of purification by means of rate-zonal sedimentation 97

Catalytic properties of the isolated DNA-dependent DNA polymerase. 104

XUM

Template specificity of the isolated DNA-dependent DNA polymerase 110

Properties of the DNA synthetic reaction stimulated by RSV RNA 113

Chromatography of the RSV-DNA polymerase 113

D. Discussion 121

References 132

XUM

Preface

The viruses and bacteriophages which employ RNA as the repository for their genetic information represent oddities in the biological scheme of things. By their exception, in complexity and genome, however, the RNA phages have contributed numerous examples which have served to strengthen rather than weaken the fundamental precepts of molecular biology. And, in the unfolding story of the alternative means by which the RNA viruses have come to express and propagate their genetic information, these entities are emerging as the molecular biologist's Galapagos.

The following presentation has been divided into two independent sections because the nature of the experimental work was quite distinct for each. The experimental evidence of each section is accompanied by an introduction where relevant literature is cited concerning experiments leading to this work, and a discussion, in which the conclusions to be drawn from these experiments are integrated with the findings of others. The first section is concerned with the characterization of the products synthesized by the poliovirus RNA-dependent RNA polymerase and the role played by membranous structures in the function of this enzyme. The second section focuses on the catalytic parameters of the DNA polymerase of Rous sarcoma virus, its isolation from viruses, and its DNA and RNA template specificity. The two sections have a common bibliography.

XUM

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Abbreviations

AcD - actinomycin D

ALV - avian leukosis virus

AMV - avian myeloblastosis virus

ATP - adenosine triphosphate

CTP - cytosine triphosphate

DEAE - diethylaminoethane

dGTP - deoxyguanosine triphosphate

DNase - deoxyribonuclease

DOC - sodium deoxycholate

GMP - guanosine monophosphate

GTP - guanosine triphosphate

moi - multiplicity of infection

NP$_{40}$- Non-idet P40 (Shell Chemical)

PCA - perchloric acid

PEP - phosphoenolpyruvic acid

PFU - plaque forming unit

rA:rU- polyriboadenylic acid hydrogen-bonded with oligoribouridylic acid

rA:T - polyribouridylic acid hydrogen-bonded with oligodeoxyribothymidylic acid

RF - replicative form RNA

RI - replicative intermediate RNA

RNase - ribonuclease

RSV - Rous sarcoma virus

SDS - dodecyl sodium sulfate

SS - single-stranded poliovirus RNA

TMP - thymidine monophosphate

Tris - Tris (hydroxymethyl) aminomethane

TTP - thymidine triphosphate

^3H UMP - tritiated uridine monophosphate

UTP - uridine triphosphate

VSB - virus synthesizing body

XUM

The RNA-Dependent RNA Polymerase of Poliovirus and the RNA and DNA-Dependent DNA Polymerase of Rous Sarcoma Virus

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The products of the membrane-associated RNA polymerase induced in HeLa cells by infection with poliovirus have been analyzed by means of polyacrylamide gel electrophoresis. In this RNA synthesizing system, endogenous RNA directs the addition of nucleotides into pre-existing RNA species. The electrophoretic profiles of the resulting RNA species are similar to those obtained with poliovirus-specific RNA species extracted from infected cells, implying that the three peaks represent, in order of increasing electrophoretic mobility, multi-stranded replicative intermediate (RI), double-stranded replicative form (RF), and single-stranded viral RNA (SS). This order has been verified by means of the characteristic behavior with respect to precipitability in 1.0 M NaCl and/or sensitivity to limited ribonuclease digestion of each of the molecular species. Kinetic studies including fate of labeled species after a "chase" have provided unequivocal evidence that RI is a functional intermediate in that it is the immediate precursor of both RF and SS species of RNA.

RNA synthesized in the presence of the detergent sodium deoxycholate (DOC) has also been analyzed by this technique, and has been shown to be distinctive in that all of the labeled nucleotides are ultimately found in RF while SS RNA is absent at all times of incubation. By adding DOC to polymerase reactions at a time when RI, RF and SS are all present,

it can be demonstrated that the SS RNA is degraded at a rapid rate, while RI is degraded at a lesser rate. Concomitant studies on the rate of degradation of purified poliovirus RNA added to reactions containing or lacking DOC suggests that DOC functions *in vitro* to free virus-specific nucleic acids of membrane association, thus rendering them accessible to endogenous ribonucleases. RNA synthesized in the presence of 3 mM guanidine-HCl was also analyzed at different times of incubation. The resulting electrophoretic profiles were found to be indistinguishable from those of control reactions.

The DNA polymerase associated with Rous sarcoma virus (RSV) has also been studied. The optimal conditions for assay have been found to be similar to those for other DNA polymerases except that non-ionic detergent is required to elicit activity. It has been ascertained that in addition to the reaction directed by endogenous RNA, activity can also be elicited by the addition of heterologous DNA. Calf thymus DNA at saturating concentrations (ca. 200 μg/ml) effects a 10-fold increase in the rate of DNA synthesis. This DNA-dependent synthesis is sensitive to high concentrations of actinomycin D.

The DNA-dependent activity has been partially purified by means of ammonium sulfate precipitation of detergent-disrupted virions followed by rate-zonal sedimentation in density gradients of glycerol. The polymerase activity sediments free of the major virus-specific polypeptide and glycopeptide components with a sedimentation coefficient of 7.1 S. The DNA-dependent activity isolated free of virus behaves identically to that associated with virions by all criteria tested. Moreover, the purification scheme simultaneously frees the enzyme of the endogenous RNA and the endogenous ribonuclease activity as judged by the absolute

requirement of added nucleic acid for activity and by resistance to degradation of RNA incubated with the isolated polymerase preparation. RSV RNA, and to a lesser extent, poliovirus RNA serve to direct the synthesis of DNA by the isolated DNA polymerase. The activity profiles of the DNA-directed and RNA-directed activities in gradients of glycerol are coincident, implying that both activities reside within a single protein component.

Part I
The RNA-Dependent RNA Polymerase of Poliovirus

Introduction

The emergence of the concept in the late 1950's and early 1960's that the synthesis of RNA takes place on DNA templates in the nucleus of eucaryotic cells left unexplained the means by which the small RNA viruses (picornaviruses) replicate their genomes. Assuming that the genetic information of the parental RNA genome must be transcribed into progeny genomes by mechanisms obeying the base-pairing rules of Watson and Crick, the question was reduced to one of whether the nucleic acid (minus strand) which serves to direct the synthesis of progeny RNA genomes (plus strand) is represented by DNA or by RNA.

Before this question concerning picornavirus replication was resolved by detection of the intermediate RNA structures and the responsible replicative enzyme(s), several lines of evidence deemed it unlikely that the minus strand is DNA. Horne and Nagington (1959) had published an electron micrographic study demonstrating the presence of cytoplasmic membranous structures associated with poliovirus virions, which suggested that poliovirus replication is a cytoplasmic process. Direct evidence against involvement of DNA in poliovirus replication was provided by Simon (1961) who showed that replication is insensitive to inhibitors of DNA synthesis. The findings that actinomycin (Reich et al., 1961 and 1962; Shatkin, 1962) and mitomycin (Reich and Franklin, 1961) do not affect virus-specific RNA synthesis at concentrations sufficient to eliminate host cell DNA-dependent RNA synthesis also offered supportive evidence against this involvement. If, as these experiments strongly suggested, the minus strand is not represented by DNA, the alternative would be an RNA minus strand.

At that time a great deal of knowledge had already been accumulated on enzymes of mammalian and bacterial origin which are capable of utilizing DNA as template for the synthesis of complementary DNA (Lehman et al., 1958) and RNA (Hurwitz et al., 1960; Weiss, 1960; Stevens, 1960). These enzymes were all shown to have similar catalytic properties, the principal features of which are the following: 1) The substrates are the nucleoside triphosphates; nucleoside diphosphates cannot be utilized as such. 2) Nucleoside monophosphates are incorporated into internal phosphodiester bonds with stoichiometric elimination of pyrophosphate. 3) The reaction is reversible; i.e. if reaction conditions are altered by removing substrate nucleoside triphosphates and adding inorganic pyrophosphate, sequential phosphorolysis of terminal nucleic acid nucleoside monophosphates occurs, resulting in product nucleoside triphosphates. 4) There is a stringent requirement for divalent metal ion--magnesium, or less often, manganese. 5) The polymerization reactions require the presence of DNA, which serves as template for synthesis of either product DNA or RNA, as the case may be. This template DNA dictates which substrate nucleoside triphosphates will be required. Native DNA templates require all four deoxyribo- or ribonucleoside triphosphates; synthetic DNA templates require only the triphosphates complementary to those bases constituting the template. This requirement suggests that the product represents a faithful complementary transcript of the base sequence of the template nucleic acid. (For reviews see Weiss, 1962: Grunberg-Manago, 1962).

Thus, the thesis that all RNA synthesis in uninfected cells takes place on DNA templates in the nucleus, the ~~evidence~~ that picornavirus replication takes place in the cytoplasm by a process which is independent of DNA, and the striking similarities in the catalytic properties of other enzymes responsible for the synthesis of biologically meaningful nucleic acids, all contributed to the rationale of Franklin and Baltimore to search in the cytoplasm of infected cells for an actinomycin resistant RNA polymerase requiring divalent cations and all four ribonucleoside triphosphates for activity. The soundness of this rationale was borne out by the detection of just such an activity in mengovirus infected L-cells (Baltimore and Franklin, 1962; 1963). This activity was shown to have a kinetics of appearance after infection similar to that of virus-specific RNA and to incorporate ribonucleoside monophosphates in a non-random fashion into ribonuclease or alkali-sensitive internal phosphodiester linkages. The failure of ribonuclease to completely inhibit the reaction precluded a positive statement regarding the template specificity of the enzyme. However, the substrate requirements of this enzyme and its resistance to DNase and actinomycin D, qualified it as a new RNA polymerase unique to picornavirus infected cells.

Another feature of the enzyme described by Baltimore and Franklin is that it can be isolated as a component of the microsomal-mitochrondrial membrane fraction from cytoplasmic extracts. This observation represented yet another source of evidence implicating membranous cytoplasmic structures as the site of

XUM

virus replication. A contemporary investigation by Becker et al. (1963) which was focused upon the immediate fate of labeled viral precursors added to cells which are actively synthesizing only poliovirus-specific components (i.e. pretreated with actinomycin D), provided evidence that these labeled precursors are quickly incorporated into rapidly sedimenting cytoplasmic structures. Furthermore, it was shown that labeled amino acids can be released as virus-specific RNA upon addition of sodium deoxycholate (DOC). With longer periods of labeling, the radioactive amino acids released by deoxycholate can also be detected in structures with sedimentation values corresponding to virions and virus capsids. These findings were in marked contrast with similar experiments conducted in uninfected cells in which labeled uridine is incorporated into the nucleus and labeled amino acids into free polysomes.

So, the early electron micrographs of Horne and Nagington, the "pulse" labeling *in vivo* studies of Becker et al, and the properties and location of the RNA-dependent RNA polymerase delineated by Baltimore and Franklin, all suggested a sequestration or compartmentalization of the major elements involved in production of progeny virions--virus-specific RNA and protein synthesis, capsid formation and virion assembly--into large membrane-bound cytoplasmic vesicles. The latter two lines of evidence were correlated in a study by Baltimore (1964), in which sedimentation experiments similar to those of Becker et al. were conducted with the variation that the partitioning between supernatant and pellet fractions versus time was determined for the poliovirus RNA polymerase activity rather than for ^{3}H uridine. The fact that the

XUM

two sets of results were virtually identical represented strong evidence that both groups of investigators had been concerned with the same structure, which was designated as the virus synthesizing body (VSB).

Baltimore used this association of the polymerase with the VSB to advantage by employing centrifugation as a means of separating the enzyme activity from the soluble cytoplasmic constituents (Baltimore, 1964). Moreover, he analyzed the extracted RNA products of the enzyme prepared in this manner by rate-zonal sedimentation, by means of which the product was separated predominantly into 35S and 16S components. This was a particularly significant finding because the 35S value corresponds to the S value of genome RNA extracted from purified poliovirus, while genome RNA hydrogen-bonded to an RNA species with complementary base composition has a sedimentation velocity of 16S. (This intracellular form of virus-specific RNA had been discovered only a short time previously by Montagnier and Sanders (1963), and its identification represented decisive evidence that RNA genomes are replicated by means of a complementary or minus RNA strand).

By carrying out the polymerase assay for varying lengths of time before extraction of the RNA product, Baltimore ascertained that the 16 and 35S components accumulate in parallel fashion, except at the earliest times when the 16S and 35S components are obscured by a heterogeneous component having S values that span the entire 16-35S region. This observation points up a shortcoming of the technique of rate-zonal sedimentation, for it subsequently became apparent that this heterogeneous component which

Baltimore was observing is the multi-stranded replicative intermediate, which can be detected as a discrete virus-specific intracellular molecular species by other means of analysis. Because of its confusing distribution in sucrose gradients, it remained for Fenwick et al. (1964) to identify and propose a functional role for this component in bacteria infected with bacteriophage R-17. The partial ribonuclease sensitivity of RI and its transient nature in pulse-chase experiments led Fenwick, Erickson and Franklin to the conclusion, which has since been amply confirmed, that this molecular species consists of a double-stranded, RNase resistant core from which RNase sensitive progeny, single-stranded RNA species are sequentially displaced.

If Baltimore had conducted such a pulse-chase experiment with his early product synthesized in vitro, he would have found the labeled nucleotides to be displaced from this heterogeneous component into the 16S and 35S molecular species. That these pulse-chase experiments were not conducted until several years later was unfortunate, because they represent the primary contribution that the isolated picornavirus RNA polymerase preparations have made to the elucidation of the molecular events of viral RNA replication. The explanation for this limitation is that, to date, no such polymerase from picornavirus-infected mammalian cells has been rendered template-dependent. Thus, information concerning molecular events during replication is limited in such systems to that which can be gained from the incorporation of labeled ribonucleotides into pre-existing species of RNA.

The only such enzyme from any source which has served to illuminate the process of RNA replication is the replicase induced by the Qβ bacteriophage. Haruna et al. (1963) originally isolated a replicase free of host cell RNA polymerases and ribonucleases from MS-2 bacteriophage, but later redirected their efforts towards the Qβ replicase, due to its unique stability (Haruna and Spiegelman, 1965). In a remarkable series of experiments Spiegelman and his co-workers demonstrated that: 1) the Qβ replicase has an absolute RNA requirement, 2) this requirement can only be met by intact Qβ RNA among natural RNA molecules, 3) the enzyme is capable of net synthesis of infectious RNA, 4) if RNA derived from a conditional lethal (ts) mutant of the virus is employed as the template, the mutant character is faithfully replicated, 5) RNA can be followed into RF and then RI (which represents an in vitro analog to the eclipse stage of virus infection because phage-specific RF and RI are not infectious in bacterial spheroplasts), and 6) the kinetics of incorporation of ribonucleotides into RF, RI and single-stranded progeny RNA can be simultaneously quantified utilizing polyacrylamide gel electrophoresis. The subunit structure of this enzyme has also recently been elucidated and shown to consist of three host-specific polypeptides and one virus-encoded polypeptide (Kamen, 1970; Kondo et al., 1970).

In contrast, virus-specific RNA polymerases isolated from mammalian cells have not, to date, been dissociated from their endogenous template and with possibly two exceptions, all require membranous association for optimal activity. Girard et al. (1967) identified a polymerase containing replicative structure which was released from poliovirus-synthesizing bodies upon solubilization of these structures with a mixture of an anionic (sodium

XUM

deoxycholate) and a nonionic detergent. The enzyme activity however, was markedly decreased, and at no time displayed linear kinetics of incorporation. The enzyme-RNA complex sediments at 250S, and was shown to be made up of the RNA-dependent RNA polymerase and an RNA component which, after extraction, sediments like RI. Making use of varying ^{3}H uridine labeling periods prior to extraction of the complex, it was shown that this structure quickly achieves a steady state of labeling while incorporation of ^{3}H UMP into total virus-specific RNA increases linearly with time. These results, in conjunction with an earlier report (Baltimore and Girard, 1966) showing that RF accumulates throughout infection while RI attains a steady state, were suggestive evidence that the RI does serve as an intermediate while RF might be a by-product of synthesis. Confirmation of the intermediate nature of RI might have been obtained by adding unlabeled uridine to the cells, but this experiment is not possible in intact mammalian cells, due to their large intracullular pools of precursor nucleotides, and to the fact that these pools exchange poorly with exogenous nucleosides.

These difficulties were circumvented by Plagemann and Swim (1968) who made use of the partially purified polymerase from mengovirus-infected Novikoff hepatoma cells, and of certain physical and chemical properties of RI, RF and SS in order to conduct such a pulse-chase study. They found that low melting RNase-resistant RNA was labeled most rapidly, and that this label could be followed into single-stranded and RF RNA species. In a similar study in which more conventional, though still indirect means were utilized for identifying the molecular species of RNA, Girard (1969) ascertained that after addition of unlabeled nucleotide precursors, label is virtually completely displaced from the RI into single-stranded genome RNA and to a lesser extent, into RF.

There is evidence from one laboratory that the enzymes responsible for the single-stranded and the double-stranded RNA species are physically separable. Arlinghaus and Polatnik (1969) used a combination of dextran sulfate and DOC to effect solubilization of foot and mouth disease virus RNA polymerase while inhibiting the endogenous ribonucleases. The enzyme-RNA complex was then analyzed by sedimentation through gradients of sucrose. Polymerase from the 100-300S region of the gradient was found to synthesize genome RNA while that from the 20-70S region synthesizes RF. It was concluded that distinct and separable enzymes are responsible for the synthesis of RF and genome RNA species. It should be kept in mind, however, that the sedimentation characteristics of the enzyme complex depend primarily on the RNA moiety. One trivial explanation of these results is that the properties of the associated RNA, which govern the sedimentation characteristics of any given complex, also govern the character of the RNA labeled *in vitro*. Thus, RNA from complexes in the 20-70S region, being primarily double-stranded, may necessarily serve as a template for the incorporation of additional nucleotides into product RNA with the physical and chemical characteristics of RF. This alternative explanation serves to point up the inescapable fact that the nucleic acid serving as template for nucleic acid-dependent nucleic acid polymerases must be considered as a factor in all aspects of the enzymology of these enzymes.

Ehrenfeld et al. (1970) have also succeeded in isolating the poliovirus RNA polymerase by washing infected cell lysates with a mixture of BRIJ and DOC, which releases the polymerase as part of a 70S template-associated complex. RNase-sensitive as well as RNase-resistant RNA is synthesized by this enzyme preparation, but the reaction proceeds for only about 20 minutes and apparently fails to exhibit zero order kinetics.

A shortcoming of the foregoing studies on the products of the partially purified polymerase from mammalian cells is that all have used indirect means of distinguishing RI and RF (i.e. RNase-sensitivity and solubility in 1.0M NaCl or LiCl). In the elegant studies of Pace et al. (1967a and 1967b) this objection was bypassed through use of the method of electrophoresis of the product RNA in gels of polyacrylamide. This procedure separates the RNA products into three molecular populations in a single step. The method has been adapted for the analysis of the corresponding but considerably larger RNA species from poliovirus infected cells (Noble et al., 1969; Noble and Levintow, 1970). In experiments to be documented below, gel electrophoresis has also been employed to characterize the RNA products of the partially purified poliovirus RNA polymerase, in order to more precisely define the catalytic functions of this template-associated enzyme, and to delineate the functional role of the membranous components isolated with this enzyme. Much of this work has been published[1,2].

1. McDonnell, J.P. and L. Levintow. 1970. Kinetics of enzymatic synthesis of poliovirus RNA. Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29, 905.

2. McDonnell, J.P. and L. Levintow. 1970. Kinetics of appearance of the products of poliovirus-induced RNA polymerase. Virol. 42, 999.

XUM

Materials and Methods

Cells and Virus. Strain S3 HeLa cells were propagated by maintenance in logarithmic phase of growth at densities of 2.0 x 10^5 cells/ml in suspension culture using spinner medium (Joklik) modification of Eagle's minimal essential medium (Grand Island Biological Co.), supplemented with 7.5% calf serum (Grand Island Biological Co.). Cells to be infected were sedimented for 4 minutes at 600 x g, washed in 0.25 volumes of pre-warmed serum-free medium, and resuspended in pre-warmed serum-free medium at ca. 10^7 cells/ml. After 10 minutes, the cell density was determined, and the cells were infected at 40 PFU/cell with a Mahoney strain type I poliovirus inoculum, prepared as a clarified lysate of cells infected overnight with poliovirus. These poliovirus stocks had titers of from 5 x 10^8 to 2 x 10^9 PFU/ml. The infected cells were diluted with serum-free medium to 5 x 10^6 cells/ml and incubated in closely stoppered vessels at 37° in a waterbath.

Preparation of particulate poliovirus RNA polymerase. This procedure was that of Baltimore (1964) with some modifications. In all experiments other than those designed to study the induction of virus-specific RNA polymerase, the cells were harvested 4 hours after infection by sedimentation at 600 x g for 4 minutes, washed in an equal volume of 0.06M KCl, 0.0015M $MgCl_2$, and 0.1M Tris-HCl, pH 7.4, and again sedimented at 600 x g for 4 minutes. The pellet was resuspended at 2 x 10^7 cells/ml in 0.01M KCl, 0.0015M $MgCl_2$, and 0.01M Tris-HCl, pH. 7.4. After 5 minutes at 0°, the cells were ruptured in a Dounce homogenizer precalibrated to maximize cell membrane vis-à-vis nuclear membrane disruption.

Cell debris and nuclei were removed by centrifugation at 600 x g for 5 minutes, and the supernatant fluid was then centrifuged at 30,000 x g for 20 minutes. The resulting pellet was resuspended with a loose-fitting Dounce homogenizer at a concentration of 10 mg of protein/ml in 0.11M Tris-HCl, pH 8.0, 0.0015M Mg(Ac)$_2$, and 0.06M KCl, and was stored at -70°.

Conditions for assay of poliovirus RNA polymerase. The standard assay mixture contained in 1.0 ml, 0.06M Tris-HCl, pH 8.0, 0.03M KCl, 0.01 Mg(Ac)$_2$, 2 μg actinomycin D, 0.25mM ATP, CTP and UTP, 0.01M phosphorenolpyruvate, 40 μg pyruvate kinase, 20 μg RNase-free DNase, 0.05mM ^{3}H GTP (0.8 Ci/mM), and 5 mg of enzyme protein. Incubation was at 37° and samples for the determination of total acid-precipitable radioactivity were withdrawn and added to 0.5 ml of 0.1M sodium pyrophosphate, and precipitated by addition of 2 ml of 3.5% perchloric acid (PCA) in 0.1M sodium pyrophosphate. After 10 minutes the precipitate was collected on a Millipore filter (0.65 μ pore size) and washed 6 times with 3 ml volumes of 0.1M sodium pyrophosphate in 1.0 N HCl. The filters were dissolved in 1 ml of 20% NH$_4$OH and counted in 10 ml of p-dioxane containing 0.5% PPO and 10% Naphthalene, at ambient temperature in a Beckman liquid scintillation spectrometer. The assay mixture was prepared by diluting the enzyme preparation into an equal volume of a solution made up by mixing the following ingredients just prior to use: 20 μg/ml actinomycin D in 0.01M Tris-HCl, pH 8.0; the three unlabeled nucleoside triphosphates at 2.5mM in 0.1M Mg(Ac)$_2$ and 0.01M Tris-HCl, pH 8.0; 0.5mM ^{3}H GTP in 0.01M Tris-HCl, pH 8.0; 5 mg/ml DNase; 8 mg/ml pyruvate kinase; and 0.1M potassium phosphoenolpyruvate dissolved just prior to use in 0.01M Tris-HCl, pH 8.0.

Extraction of RNA. RNA was extracted essentially according to Noble et al. (1969). To 0.5 ml samples of standard reaction mixtures in conical centrifuge tubes was added 0.5 ml of 0.1M NaCl, 1.0mM EDTA, and 0.01M Tris-HCl, pH 7.4 (TNE) with 0.5% SDS and 1.0 ml phenol saturated with TNE. The two phases were mixed by shaking the tube for 2 minutes at room temperature, and then separated by centrifugation at 600 x g for 3 minutes at room temperature. The aqueous phase was removed, and this extraction procedure was repeated two more times. The final aqueous phase was added to two volumes of absolute ethanol precooled to -20°, and maintained at -20° overnight. The ethanol-precipitated RNA was sedimented at 600 x g for 20 minutes at 4°, washed once with 70% ethanol in TNE at 4°, and dissolved in 0.75 ml TNE-0.5% SDS. OD 260/280 ratios for such RNA preparations varied between 1.8 and 2.1. These samples were stored at -70° without detectable degradation. Acid-precipitable radioactivity in the purified RNA was ascertained as described above for the enzyme assay.

RNase digestion of purified RNA. RNase-degraded RNA samples to be analyzed in gels of polyacrylamide could not be digested in 2 x SSC because the high ionic strength of the buffer resulted in non-reproducible electrophoretic migration of the RNA. The high NaCl requirement necessary to prevent degradation of double-stranded RNA can be avoided by substitution of 0.01M $Mg(Ac)_2$ (Bishop et al., 1965). Accordingly, RNase digestion was carried out by incubation of purified RNA samples with 5 μg/ml pancreatic

RNase in 0.01 x SSC and 0.01M $Mg(Ac)_2$ for 30 minutes at 37°.

Salt fractionation of purified RNA. Double-stranded RNA (RF) was separated from RI and single-stranded RNA by virtue of its solubility in 1.0M NaCl. Purified RNA samples were rendered 1M with respect to NaCl by the addition of 0.25 volumes of 5M NaCl and kept at -20° overnight. The RNA precipitate was sedimented at 600 x g for 20 minutes at 4°, and the supernatant containing RF was dialyzed overnight against TNE at 4°. The pellet was washed once with 1.0M NaCl and resuspended in the original volume of TNE-0.5% SDS.

Preparation of polyacrylamide gels. Gels were prepared essentially according to Noble et al. (1969). Acrylamide and bisacrylamide were recrystallized from chloroform and acetone respectively, and stored in an evacuated desiccator at 4° in the dark. A stock solution of 15% (w/v) acrylamide and 0.75% (w/v) bisacrylamide was prepared and diluted to 2.25% with respect to acrylamide by addition of electrophoresis buffer containing 0.04M Tris, 0.02M NaAc, 0.002M Na EDTA and adjusted to pH 7.8 with glacial acetic acid. This solution was degassed with an aspirator, after which 0.075% (v/v) NNN'N'-tetramethylethylenediamine and 0.075% (w/v) ammonium persulfate were added. The contents were immediately added to 6 cm. in 7 cm. silicone-coated glass tubes having an inner diameter of 0.5 cm. A water overlay was added and polymerization was allowed to proceed for at least 30 minutes at room temperature.

Electrophoresis in polyacrylamide gels. Electrophoresis was conducted essentially according to Noble et al. (1969). Electrophoresis was conducted in a vertical Canalco gel electrophoresis apparatus using electrophoresis buffer (EB). After pre-electrophoresis for 1 hour, samples of from 75-100 µg of RNA at a concentration not greater than 0.5 mg/ml in 10% sucrose and 0.5% SDS in TNE were layered on top of the polyacrylamide gels. Electrophoresis was carried out at 4° at 5 mA/gel for 3.5 hours. After electrophoresis, the gels were removed from the glass columns with a stream of water from a hypodermic syringe having a 26 gauge blunt needle. The gels were then frozen and cut into 1 mm. sections with an accurately spaced battery of razor blades (Diversified Scientific Instruments, San Leandro, California). The gel slices were digested overnight in 1.5 ml of 1 x hydroxide of Hyamine (Packard) in methanol at room temperature and were counted at ambient temperature after addition of 10 ml of 4.2% (v/v) Liquifluor (New England Nuclear) in toluene.

Protein determinations. Concentrations of protein were determined by the method of Lowry et al. (1951) against a bovine serum albumin standard.

Reagents. The sources of reagents were: actinomycin D (Merck, Sharpe and Dohme); unlabeled ribonucleoside triphosphates and phosphoenolpyruvic acid-monopotassium salt (Calbiochem); pyruvate kinase (Sigma); pancreatic ribonuclease A and deoxyribonuclease, electrophoretically purified and free of ribonuclease activity (Worthington Biochemicals, Inc.); ^{3}H Guanosine 5' triphosphate-tetra lithium salt (GTP), 0.8 to 1.2 Ci/mmole (Schwarz BioResearch); 2,5-diphenazloxazole (PPO) (New England

XUM

Nuclear Chemicals); p-dioxane, reagent grade and phenol, reagent grade (Mallinckrodt); deoxycholic acid-sodium (DOC), and dodecyl sodium sulfate (SDS) (Matheson, Coleman and Bell); and acrylamide, bisacrylamide and NNN'N'-tetramethylethylene-diamine (TEMED) (Eastman Kodak Organic Chemicals).

Results

Kinetics of appearance of poliovirus RNA polymerase. It has been demonstrated that the ongoing rate of viral RNA synthesis in cells at the time they are harvested for fractionation of the virus-specific RNA polymerase, is rather accurately reflected in the activity of the resulting enzyme preparation, (Ehrenfeld et al., 1970). Since many factors including multiplicity of infection (moi) influence a viral RNA growth curve, it is necessary to determine the time after infection at which maximal polymerase activity is achieved for any given set of conditions of infection and incubation. When cells infected as described in Materials and Methods are harvested at increasing times of incubation, polymerase activity is detectable at 2.75 hours, reaching a plateau after 3.75 hours (Figure 1). Cells incubated for 4 hours were used as the source of enzyme for subsequent experiments.

Electrophoretic analysis of the product of the viral RNA polymerase. Upon electrophoresis through gels of 2.25% polyacrylamide, the RNA labeled *in vitro* is delineated into three distinct peaks which correspond closely to species of virus-specific RNA isolated from infected cells. This correspondence is demonstrated in Figure 2 by superimposing the electrophoretic pattern of RNA labeled *in vivo* on an electrophoretic analysis of the product of the isolated polymerase. In order to verify the implication that the coincident peaks of RNA labeled *in vivo* and *in vitro* represent similar molecular species, purified RNA product extracted from the enzyme reaction was fractionated by precipitation and/or ribonuclease digested prior to electropho-

Figure 1. Rate of synthesis of enzyme prepared at increasing times after infection. Samples for preparations of enzyme were removed from an infected culture every 30 minutes starting at 2-$\frac{1}{4}$ hours after infection, and the kinetics of RNA synthesis by each in a standard assay was determined. The time after infection that each preparation was obtained, is indicated on the right.

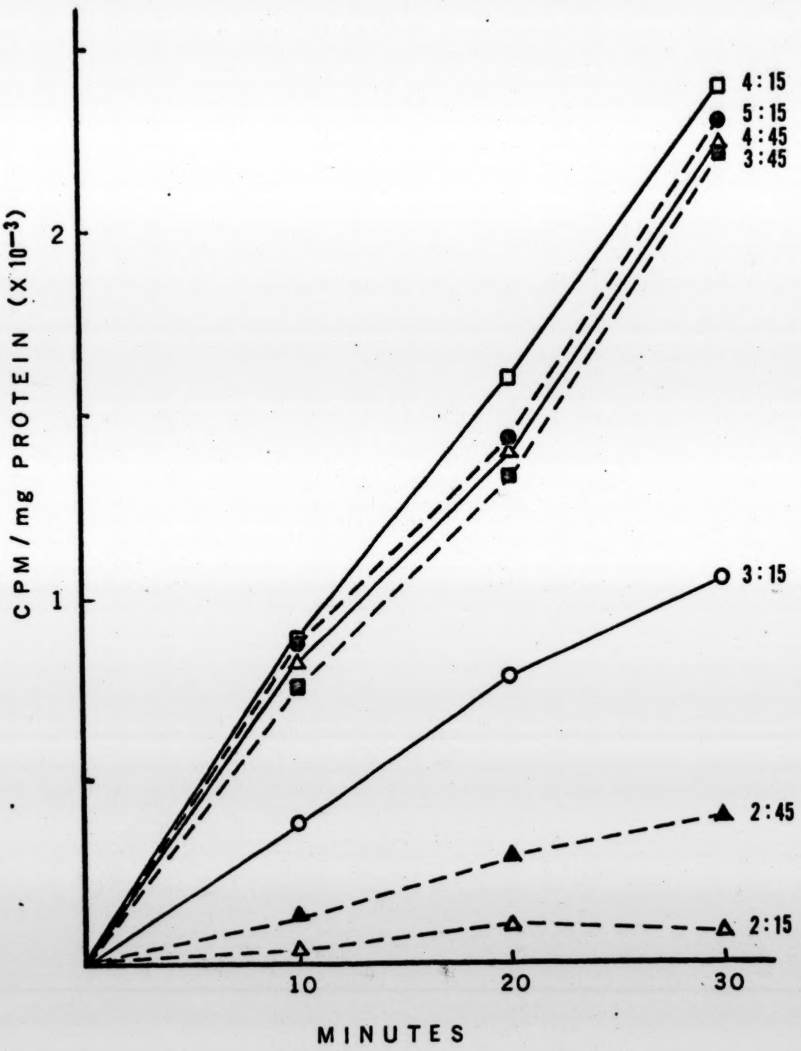
CPM / mg PROTEIN (X 10^{-3})
2
1
10
20
30
MINUTES
4:15
5:15
4:45
3:45
3:15
2:45
2:15

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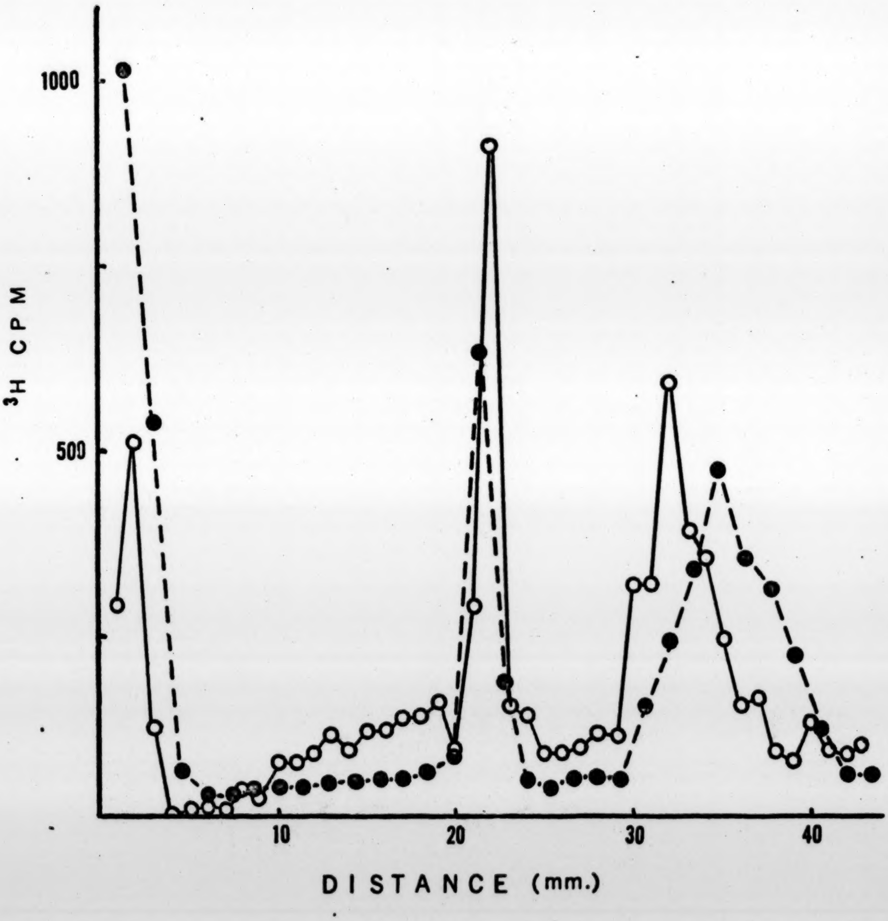
Figure 2. *Superimposed electrophoretic analyses of poliovirus-specific RNA from infected cells(●- - - -●) and the products labeled* in vitro *by crude, particle-associated RNA polymerase (○———○).* RNA was extracted at 3-1/3 hours after infection from actinomycin-treated cells labeled with 20 µCi/ml of uridine-^{3}H (Noble and Levintow, 1970), and from a standard enzyme reaction mixture at 30 minutes, and analyzed by gel electrophoresis as described in Materials and Methods. Fractions 1-4, RI; fractions 20-24, RF; fractions 29-41, SS RNA.



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retic analysis. High molecular weight single-stranded RNA is insoluble in 1M NaCl. Consequently, RF remains in solution while RI and genome RNA are precipitated under these conditions (Montagnier and Sanders, 1963). The electrophoretic profiles of the fractionated RNA product are shown in Figure 3A. The RNA recovered from the 1M NaCl supernatant migrates to the position of the middle peak, whereas the redissolved 1M NaCl precipitate is found predominately in the regions of the first and third peaks of unfractionated product RNA. If samples of this 1M NaCl supernatant RNA are subjected to ribonuclease digestion (Materials and Methods) prior to electrophoresis, the resulting profile is only slightly affected (Figure 3B). However, as can be seen in Figure 3C, ribonuclease digestion of the RNA precipitated by 1M NaCl completely abolishes the most slowly and the most rapidly migrating peaks of labeled RNA, leaving only a residuum of RNA at a position coincident with that of the NaCl-soluble (and ribonuclease resistant) RNA of Figure 3B. Similar results are obtained by observing the effect of ribonuclease digestion on unfractionated product RNA (Figure 4). It is readily apparent that the first and third peaks are both completely RNase sensitive while the middle peak is RNase resistant. These results are entirely consistent with similar experiments conducted with viral RNA labeled _in vivo_ and with purified RI, RF and virion RNA standards, which had previously established that the three peaks differentiated on polyacrylamide gels are, in order of increasing electrophoretic mobility, RI, RF and virion-like RNA (Noble et al., 1969).

It can be concluded from these experiments that the isolated

Figure 3. Electrophoresis of enzymatic product fractionated by salt and digested by RNase. Product analogous to that of Figure 2 was first fractionated by precipitation in 1.0M NaCl and then analyzed by gel electrophoresis as represented in Panel A. Panels B and C demonstrate the effect of ribonuclease digestion on the electrophoretic profiles of the supernatant and precipitate fractions respectively.

A) Precipitated RNA (○——○——○); Supernatant RNA (●- - -●- - -●).

B) Undigested supernatant RNA (●- - -●- - -●); RNase digested RNA (○——○——○).

C) Undigested precipitated RNA (○——○——○); RNase digested precipitated RNA (●- - -●- - -●).

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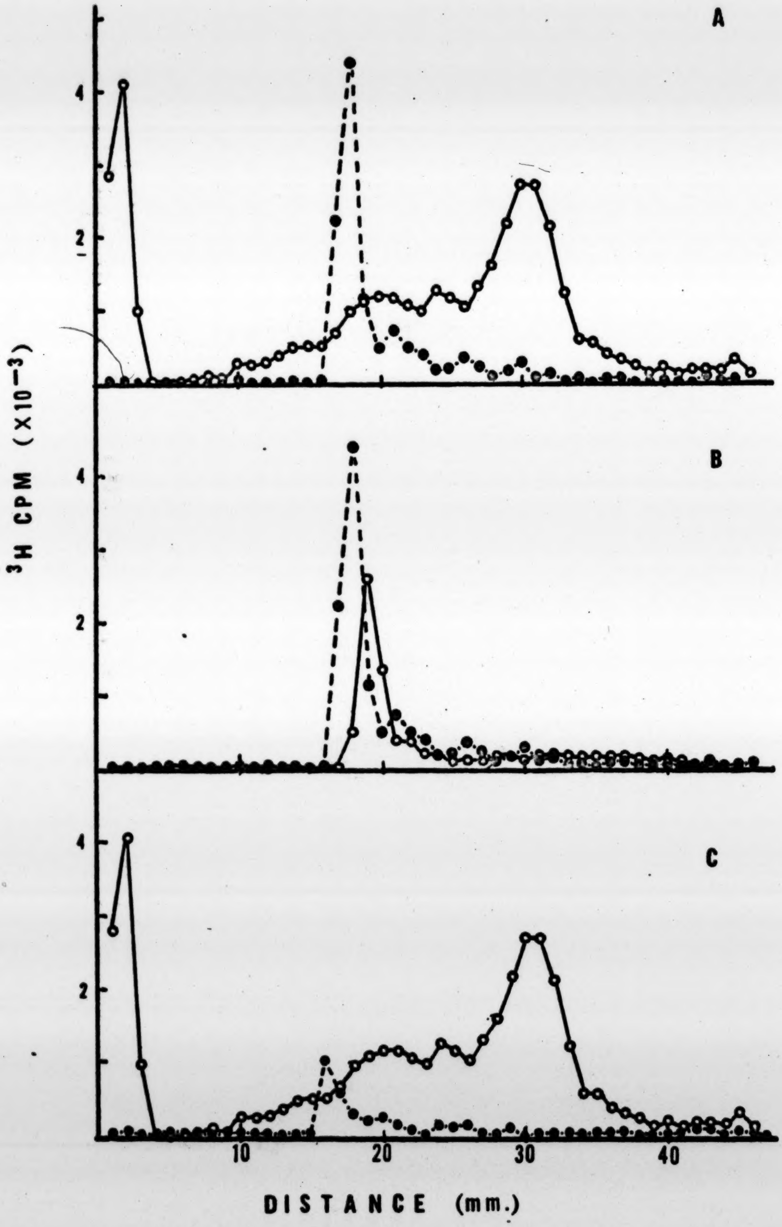

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Figure 4. Effect of RNase digestion on unfractionated enzymatic product. Equivalent portions of purified RNA from a standard reaction were either digested with ribonuclease or used as an undigested control for analysis by electrophoresis in gels of polyacrylamide. RNase digested portion (●---●---●); undigested control portion (○—○—○).

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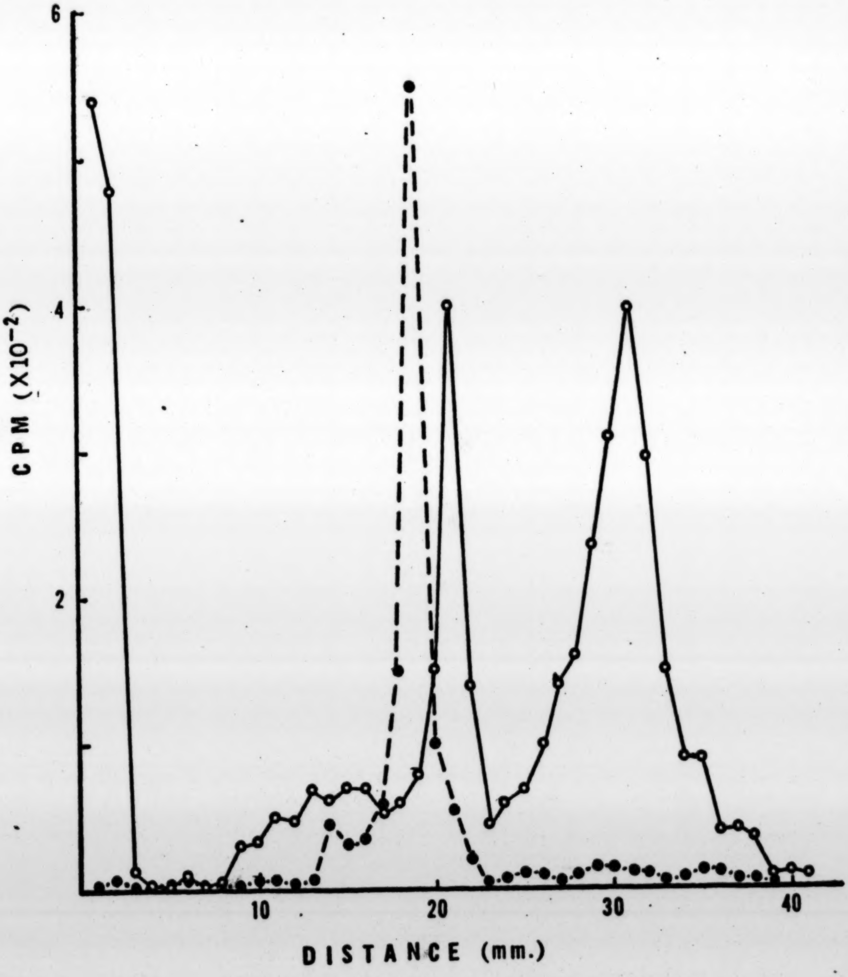

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poliovirus RNA polymerase catalyzes the incorporation of ^{3}H GMP into three distinct molecular species of RNA, and that these correspond closely to the three virus-specific RNA species obtained from infected cells with respect to RNase sensitivity, solubility in 1M NaCl, and electrophoretic mobility. Thus, the RNA peak at the origin is the multi-stranded RI, the intermediately migrating peak is double-stranded RF and the most rapidly migrating peak is single-stranded (SS) genome RNA.

Kinetics of enzymatic labeling of RI, RF, and SS RNA. The sharp delineation of the three enzymatic products by gel electrophoresis provides a convenient and unambiguous means for simultaneously determining the rate of incorporation of radioactive precursors into RI, RF and SS RNA. Such experiments were conducted by analyzing the radioactive product from assays incubated for increasing periods of time, and summing the amount of radioactivity in each of the electrophoretically separated peaks. As can be seen in Figure 5, incorporation of labeled precursors is predominantly into RI in the early part of the reaction, but by 20 minutes there is no further accumulation into this species. The incorporation of label into SS is nearly linear after an initial lag, while RF accumulates at a linear but somewhat slower rate than the other species. These initial kinetics, whereby radioactivity is found primarily in a single RNA species, represent an ideal circumstance for conducting isotope dilution experiments in order to ascertain the fate of this rapidly labeled RNA. Because the rate-limiting substrate in the standard reaction is the ^{3}H-GTP, the rate of the overall reaction can be

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Figure 5. Time course of enzymatic labeling of virus-specific species of RNA. Samples were withdrawn from an incubation mixture at the indicated times and analyzed by electrophoresis. The amounts of RI (○———○), RF (●- - - -●), and SS RNA (□———□) represent the average of three experiments.

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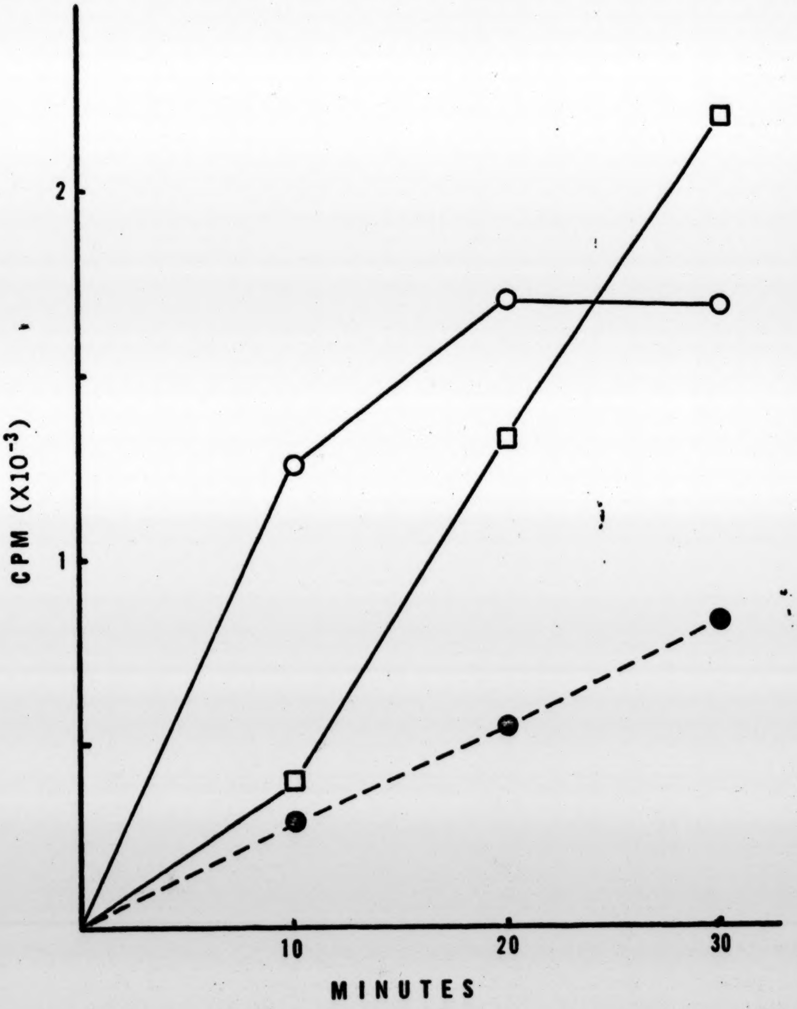

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altered simply by adjusting the concentration of this precursor. In order to expand the time course of the early part of the reaction for "chase" experiments, the ^{3}H-GTP was reduced from 50 to 12 nmole/ml. The procedure results in an approximate 2-fold reduction in the overall rate of incorporation, while the ratio of RI to either RF or SS at 15 minutes, corresponds to that expected at ca. 7 minutes of a standard reaction (compare Figure 5 with zero time point of Figure 8). A 125-fold excess of unlabeled GTP added at this time results in a virtually complete cessation of incorporation of radioactive substrate into RNA while a like concentration of unlabeled ATP has no effect on incorporation (Figure 6). Purified RNA from samples withdrawn just prior to, and at various times after addition of the unlabeled GTP, gives the series of electrophoretic profiles depicted in Figure 7. The distribution of radioactivity in these profiles was determined, and the data was employed to construct the composite plot of Figure 8. It should be noted (see Table I) that the total amount of radioactivity recovered in the three peaks at any time point is nearly constant, suggesting that all of the radioactivity is accounted for throughout the "chase." These results leave little doubt that RI serves as a precursor in the synthesis of both RF and SS RNAs.

These results also suggest an explanation for the observation that accumulation of radioactivity into RI reaches a plateau (Figure 5). If, in its function as an intermediate, RI attains a plateau because it has reached a steady-state where labeled nucleotides are leaving this species at the same rate they are being incorporated, then it should be possible to displace this

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Figure 6. Course of enzymatic incorporation of GMP-^{3}H into total poliovirus-specific RNA following a "chase." Standard incubation mixtures were prepared as described in the text except that the concentration of GTP-^{3}H was reduced to 12 mμmoles/ml. Portions (0.25ml) were withdrawn at the indicated times and analyzed for acid-precipitable radioactivity. The addition of 1.5μmoles of unlabeled GTP (○———○) is compared with the addition of a similar amount of unlabeled ATP (□———□), and no addition (●- - - - -●).

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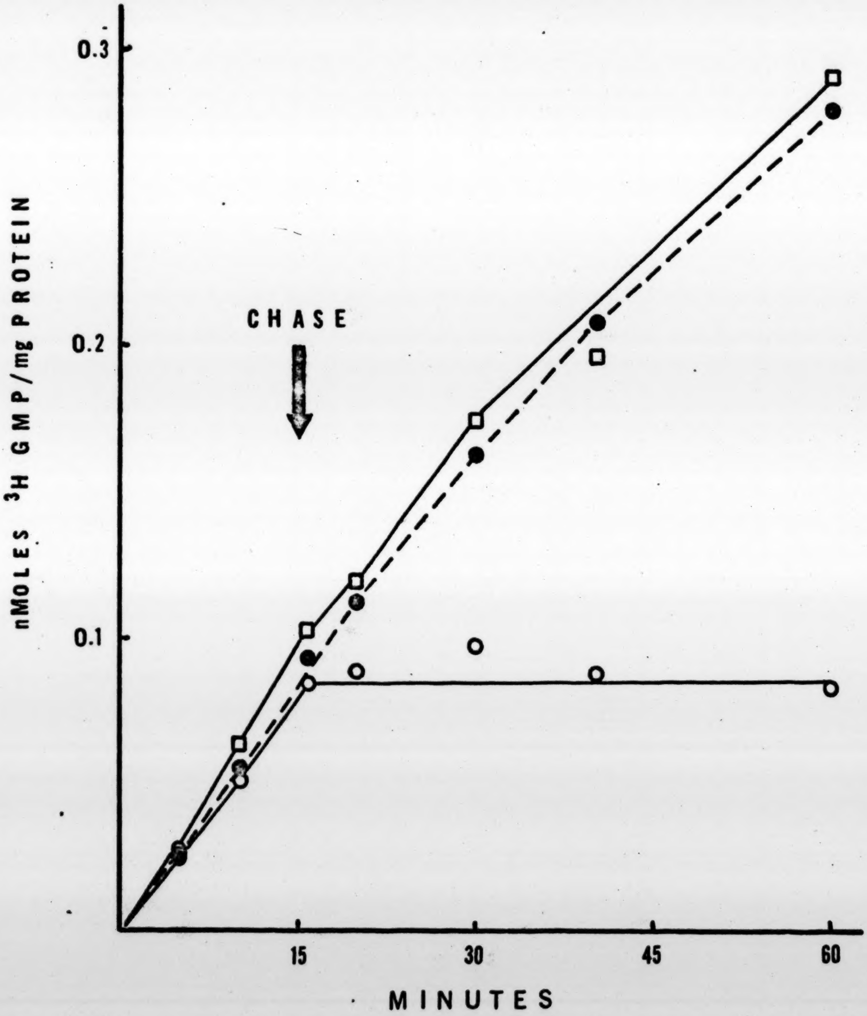

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Figure 7. Electrophoretic profiles of enzymatic product obtained immediately before (Panel A), and 2.5 and 15 minutes after (Panels B and C) the "chase" described in Figure 6.

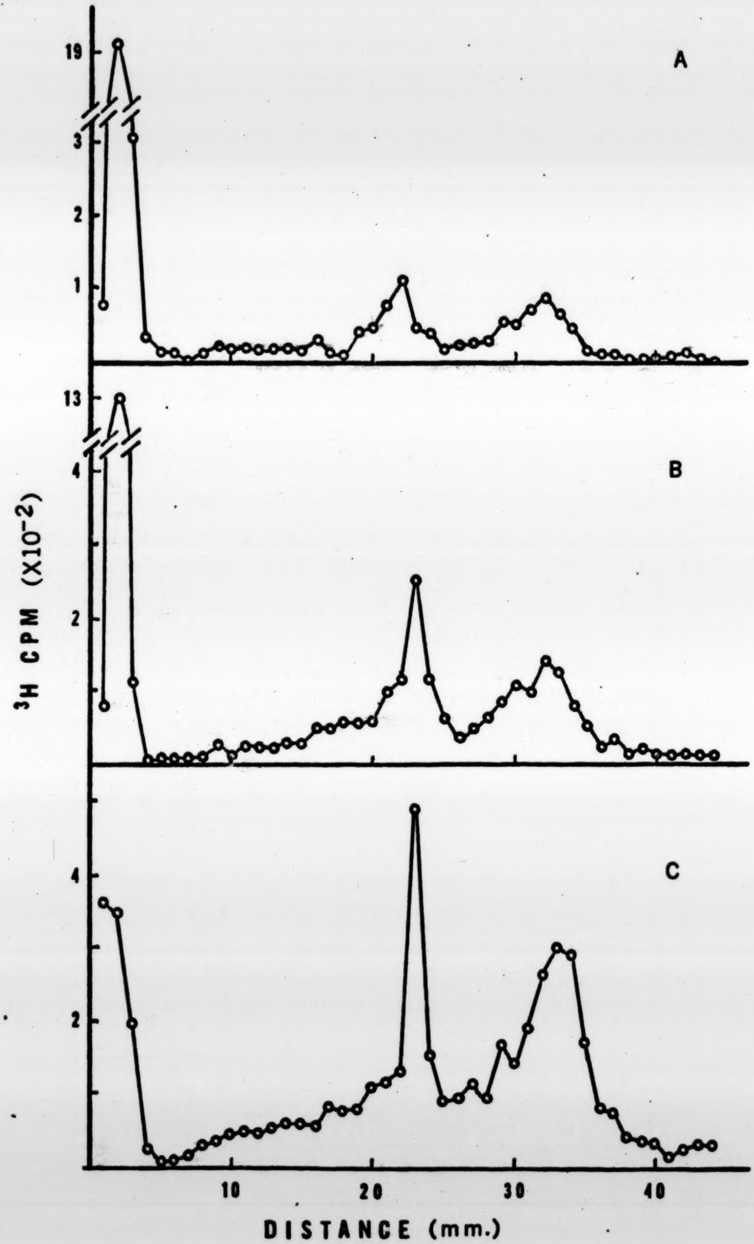

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Figure 8. Fate of enzymatically labeled poliovirus-specific RNA after a "chase" conducted at 15 minutes of incubation. Data from the electrophoretic profiles depicted in Figure 7 and other profiles not shown were used to construct this plot, which represents the distribution of the label between RI (○——○——○), RF (●- - -●- - -●), and SS RNA (□——□——□) at the indicated times after the chase.

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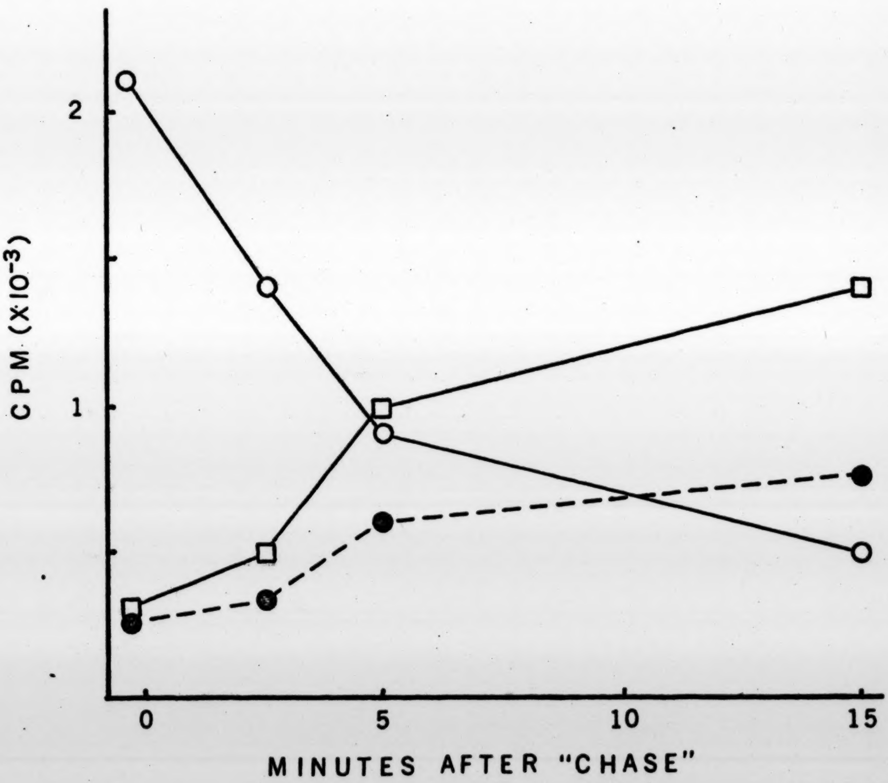

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Table I
^{3}H c.p.m. detected in the RI, RF and SS regions following a "chase."

Minutes after "chase"	"Chase" at 15 minutes	"Chase" at 40 minutes
0	2780	5500
5	2310	5500
10	2460	5300
15	2570	-
20	-	5450
50	-	5400

Data from the gel profiles of "chases" conducted at 15 minutes and 40 minutes of incubation described in Figures 7 and 9 were compiled as described in Figures 8 and 9. The total ^{3}H c.p.m. under the RI, RF and SS peaks were added to give the indicated values for each time point.

label during conditions of a "chase." A "chase" conducted at 40 rather than at 15 minutes, corresponding to a time when RI has reached a plateau in a standard reaction, does result in the displacement of nearly all of the radioactivity of this RNA species into RF and SS (Figure 9). It can be concluded that RI assayed *in vitro* attains a steady-state of incorporation of radioactive nucleotides in a fashion analogous to its counterpart from infected cells (Noble and Levintow, 1970).

Effect of DOC on enzyme function. Procedures designed to render picornavirus-induced RNA polymerase soluble and template-dependent necessarily include steps making use of agents which disrupt membranous structures. The most widely employed and successful agent for this purpose has been the anionic detergent sodium deoxycholate (DOC), either in conjunction with a nonionic detergent (Girard et al., 1967; Ehrenfeld et al., 1970), or a non-specific ribonuclease inhibitor (Arlinghaus and Polatnick, 1967). The presence of DOC results in the release of an enzyme-RNA complex having a greatly reduced sedimentation velocity from the large membranous structures. Although this enzyme fraction remains independent of added RNA for activity, the kinetics of RNA synthesis are altered (Figure 10) (Girard et al., 1967; Plagemann and Swim, 1968). In an effort to clarify the effect of DOC on the synthesis of RNA, electrophoretic analysis of the products of standard reactions containing DOC was conducted. The most striking alteration in the electrophoretic profiles of such reactions was the complete absence of single-stranded RNA at all times of assay. The time-course of enzymatic labeling of the

Figure 9. Fate of enzymatically labeled product after a "chase" conducted at 40 minutes of incubation. The data for the construction of this plot was obtained in a fashion analogous to that of Figure 8, with samples for electrophoresis being withdrawn just prior to, and 5, 10, 20 and 50 minutes after the "chase." RI (○——○——○), RF (●---●---●), and SS RNA (□—□—□).

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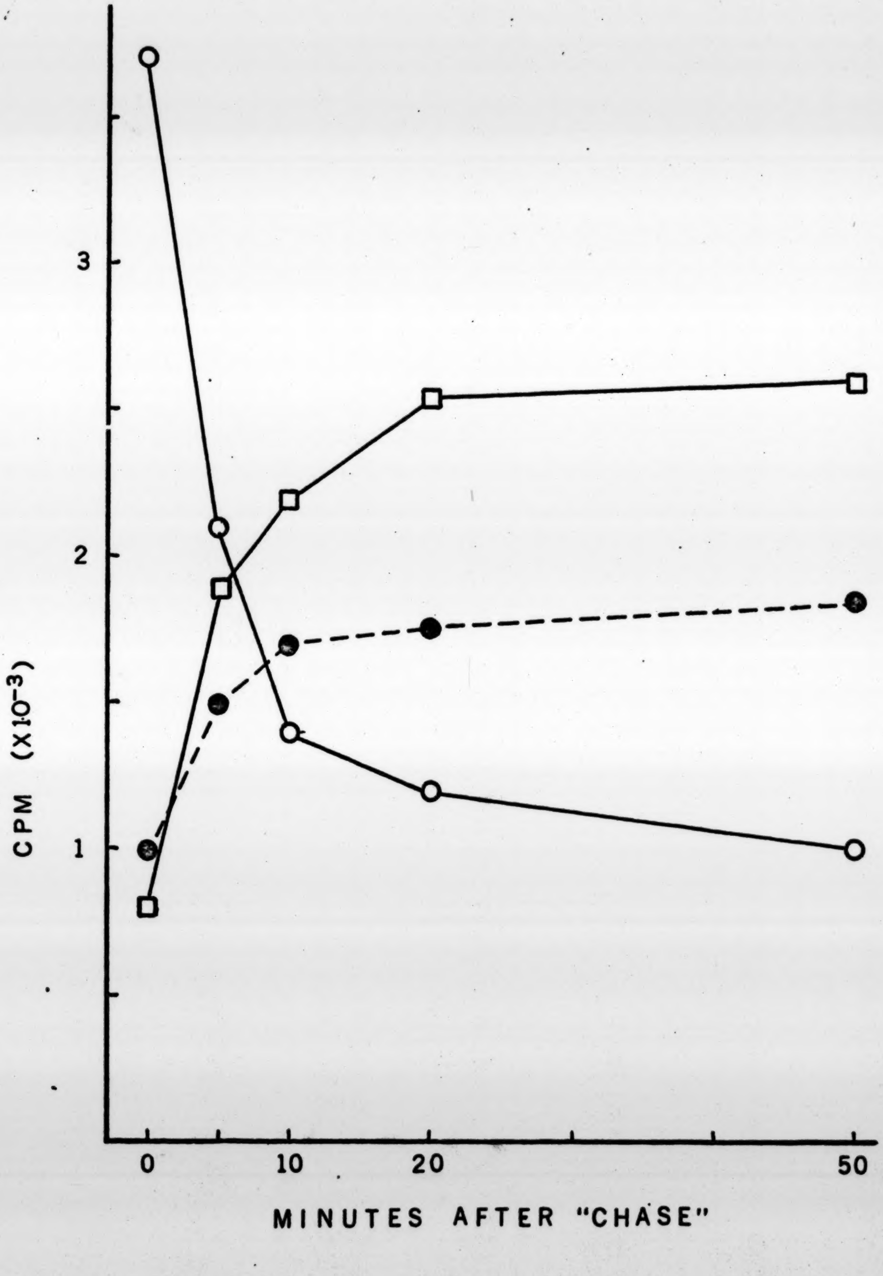

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Figure 10. Effect of DOC on kinetics of enzymatic synthesis. The rate of incorporation of ^{3}H GTP into acid-insoluble product in a standard assay versus one containing 0.5% DOC is depicted. DOC-treated (●---●---●); untreated control (○—○—○).

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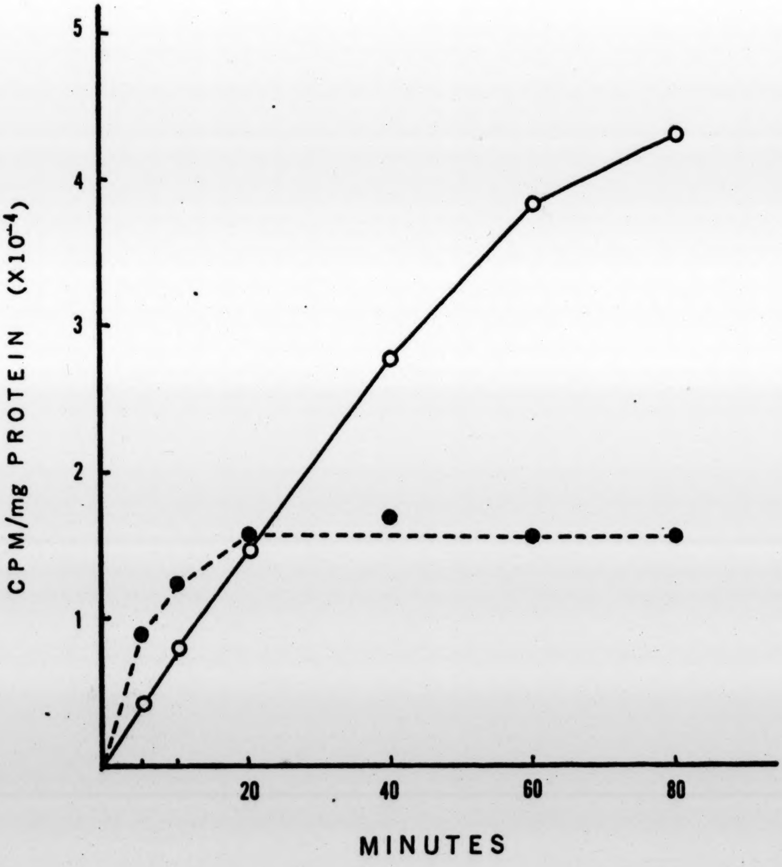

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RI and RF species of RNA are shown in Figure 11. In keeping with the overall kinetics of RNA synthesis, radioactive RNA precursors are initially incorporated at a rapid rate into RI, but by 15 minutes when net incorporation has ceased, radioactivity shifts from RI to RF. At later times, the electrophoretic profiles of this product become indistinguishable from those of ribonuclease-treated RNA from standard reactions.

The absence of SS RNA in DOC treated polymerase assays may be explicable on the basis of either a block in synthesis or an increased rate of degradation. The latter possibility was tested for by adding DOC to a standard reaction at a time when all three product RNA species are present. The electrophoretic profiles of labeled RNA obtained at various times from such an experiment are shown in Figure 12; the results are summarized in Figure 13. Clearly, single-stranded RNA species are rapidly degraded upon addition of DOC while RF continues to accumulate radioactivity. That this phenomenon is not simply due to liberation or activation of latent nucleases (e.g., by-way-of disruption of lysosomes) is shown in the following experiment. ^{32}P-labeled poliovirus RNA was added to standard and DOC-treated reactions, and the degradation of this RNA was followed simultaneously with the appearance of ^{3}H-labeled product RNA. As can be seen in Figure 14, the rate of degradation of the added ^{32}P-labeled RNA is much the same in either case. Moreover, in the reaction without DOC, the digestion of the added RNA proceeds concomitantly with the appearance of enzymatically labeled product RNA species, including SS RNA which presumably is physically identical to this ^{32}P-viral RNA (Figure 15). It would appear

Figure 11. Time course of enzymatic labeling of virus-specific species of RNA in the presence of DOC. Experimental conditions were as described in Figure 2, except for the presence of 0.5% DOC in the reaction mixture. RI (○———○); RF (●- - - -●).

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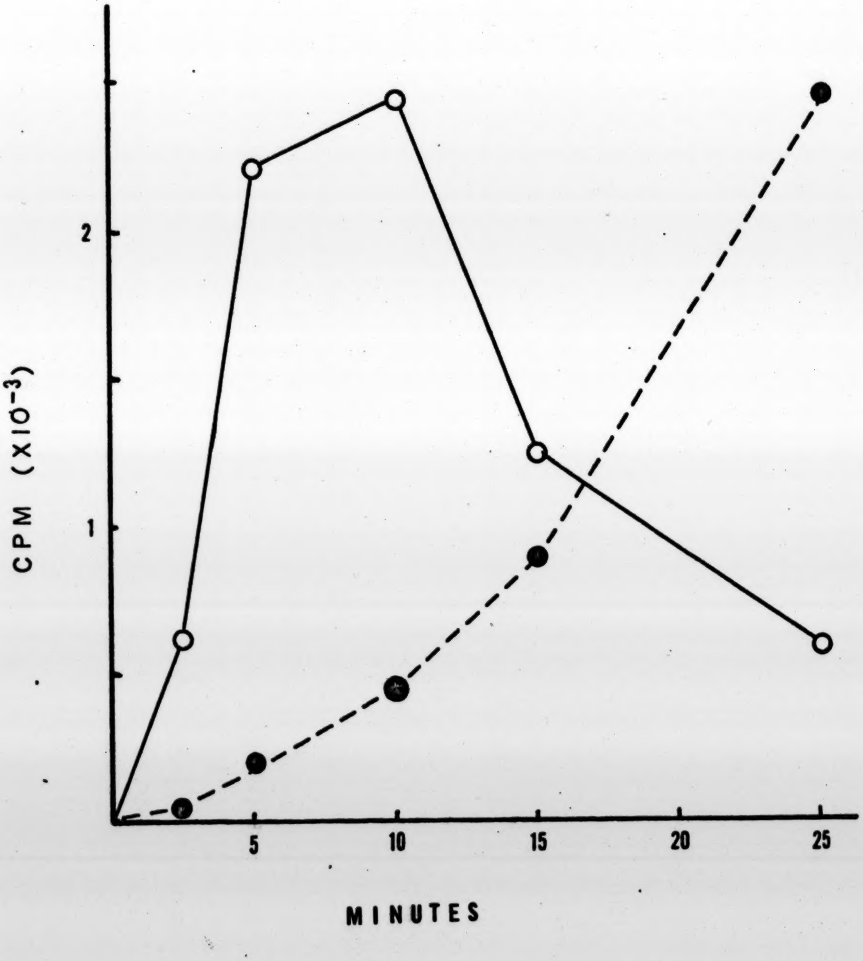

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Figure 12. Effect of DOC on enzymatically labeled species of RNA. Panels A, B, and C depict electrophoretic analyses of RNA labeled in a standard reaction mixture immediately before, 5 minutes after, and 25 minutes after making the reaction 0.5% with respect to DOC.

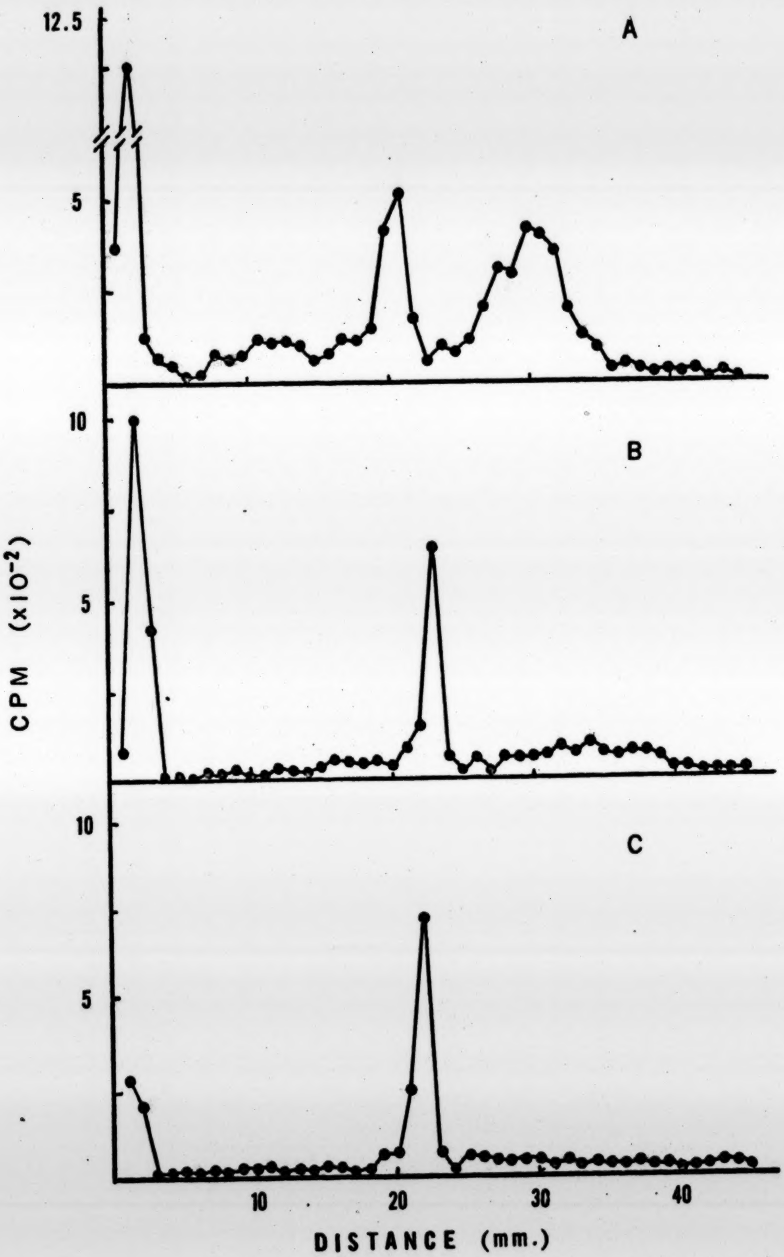

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Figure 13. Fate of enzymatically labeled product after addition of DOC. The data of the electrophoretic profiles of Figure 12 and others not shown was employed to construct this plot, which depicts the distribution of the label between RI (○—○—○), RF (●- - -●- - -●), and SS (□—□—□).

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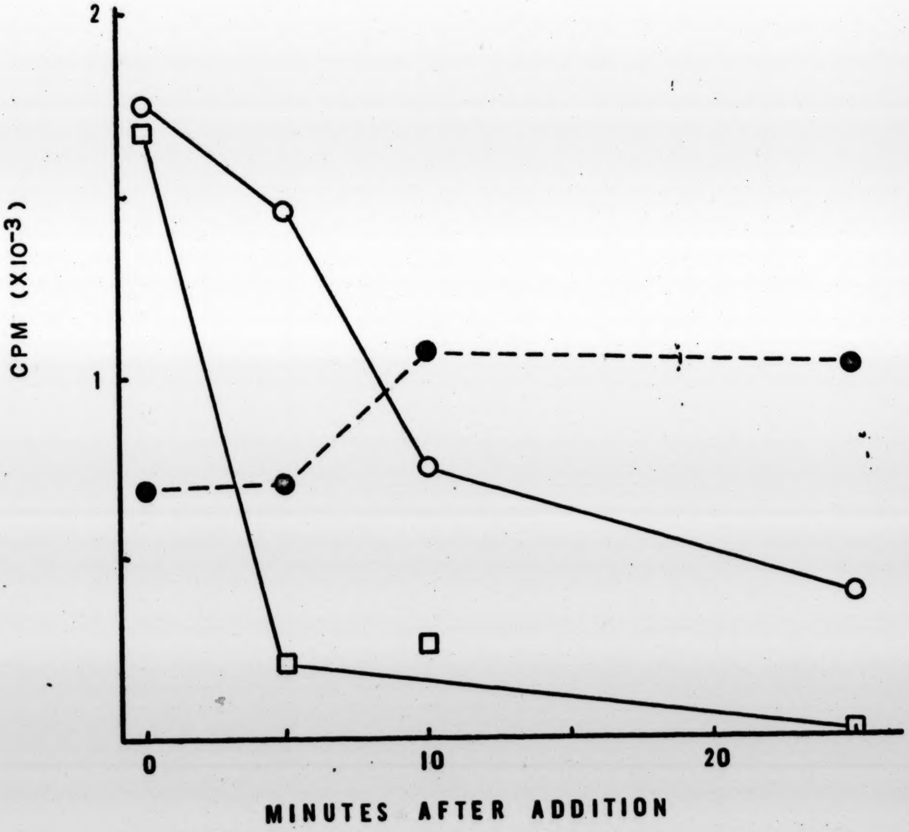
2
1
CPM (X10^{-3})
0
10
20
MINUTES AFTER ADDITION

Figure 14. Effect of DOC on the degradation of SS RNA added to digests of particle-associated RNA polymerase. ^{32}P-labeled SS RNA prepared from purified virions (3 X 10^{6} cpm in 10μl) was added to standard complete incubation mixtures in the presence (●-----●) and the absence (○———○) of 0.5% DOC, and the relative amount of intact ^{32}P-labeled SS RNA remaining was determined by electrophoretic analysis at various times.

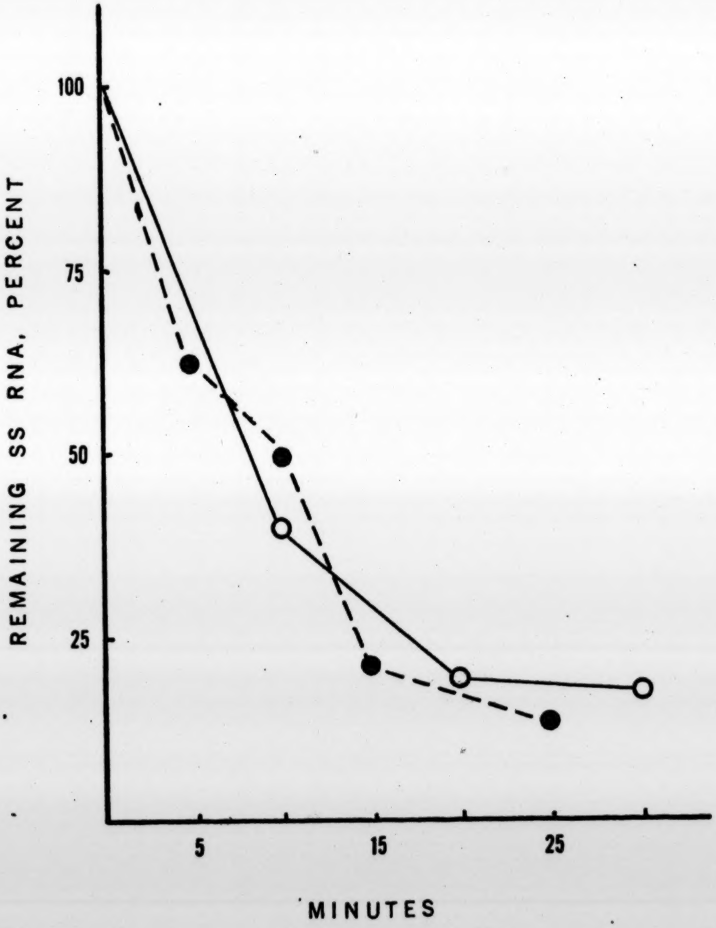

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Figure 15. Concomitant appearance and disappearance of labeled SS poliovirus RNA. Electrophoretic analyses of the reaction mixture without DOC described in the legend to Figure 7 at zero time (A), and after incubation for 30 minutes (B) are depicted. ^{32}P-labeled, added SS RNA (●——●); ^{3}H-labeled enzymatic products (○——○).

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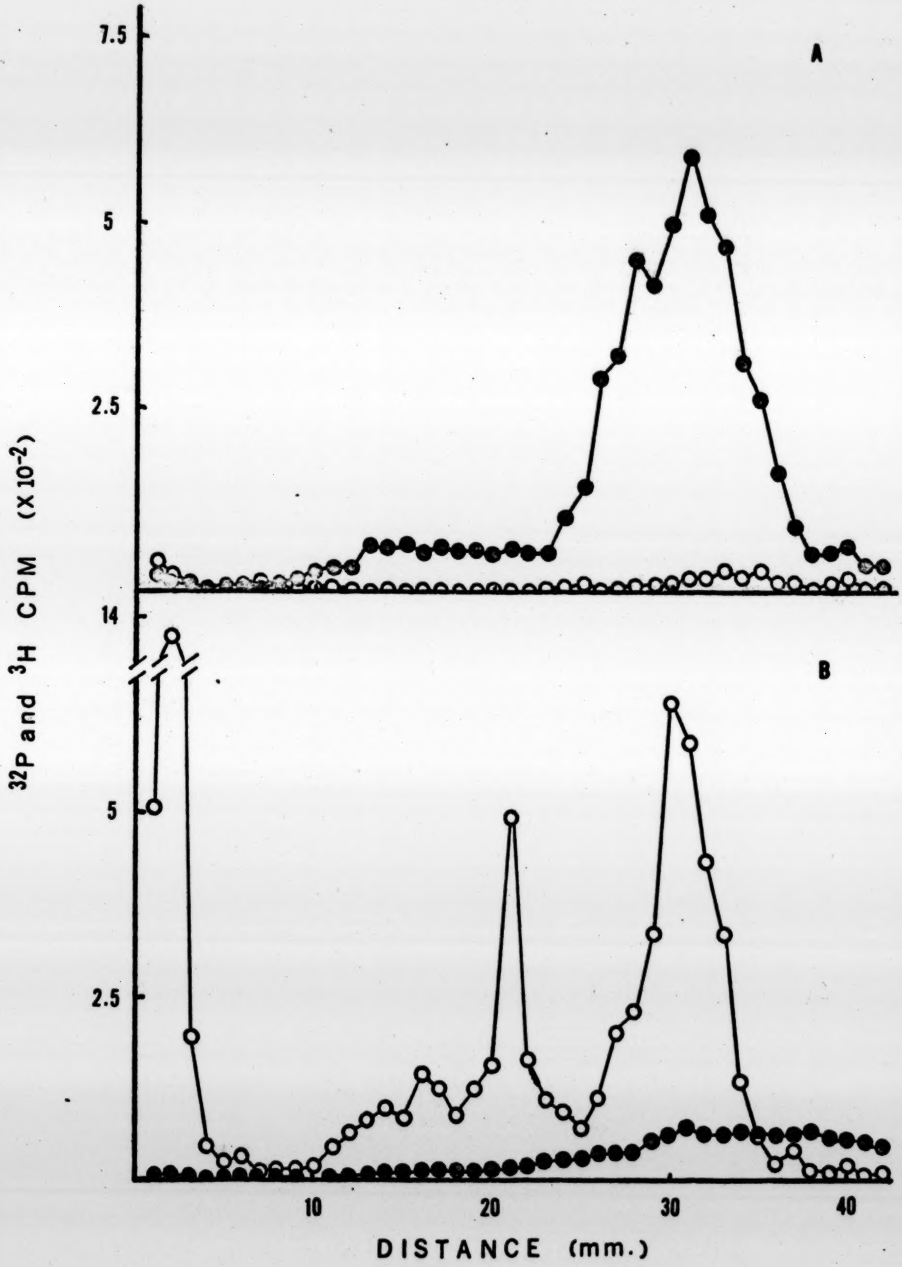

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then that active nucleases are present in the membrane-associated polymerase preparation, but that the enzymatically labeled products are sequestered in a fashion affording considerable protection from these nucleases. This view is supported by the experiment shown in Figure 16, which demonstrates that over 95% of the labeled RNA product remains associated with the particulate structures sedimentable by centrifugation for 20 minutes at 30,000 x g. In this experiment the partition between supernatant and pellet of total RNA was determined by measuring the optical density at 260 mμ. The distribution of added ^{32}P-viral RNA which had not been degraded by 30 minutes of incubation (ca. 15%) and thus continued to migrate into the SS region of the polyacrylamide gels, was also ascertained. As can be seen in Table II, partition of the ^{3}H-labeled RNA product is distinctive in the conditions of this experiment. While a more striking difference between the sedimentation of product RNA vis-à-vis total or added RNA might have been achieved by the utilization of sucrose gradients, the purpose of this experiment was simply to observe the distribution of RNA under conditions analogous to those which are known to sediment the polymerase associated with virus-synthesizing bodies.

Effect of guanidine on the products of enzymatic synthesis. It has been long known that certain picornaviruses including poliovirus are inhibited by guanidine-HCl at concentrations of ca. 1-5 x 10^{-3}M. At an early time in his studies on the poliovirus-specific RNA polymerase, Baltimore (1963) showed that guanidine at these and much higher concentrations has no effect

Figure 16. Association of the products of RNA polymerase with sedimentable particles. A standard reaction mixture after 30 minutes of incubation was chilled, and centrifuged at 30,000 g for 20 minutes at 4°. The pellet was suspended in the original volume of 0.12M Tris-HCl, pH 8.0, 0.0015M $Mg(Ac)_2$ and 0.06M KCl, and RNA was extracted from it and from the supernatant fraction. Electrophoretic analysis of the pellet (○——○); and supernatant fraction (●——●).

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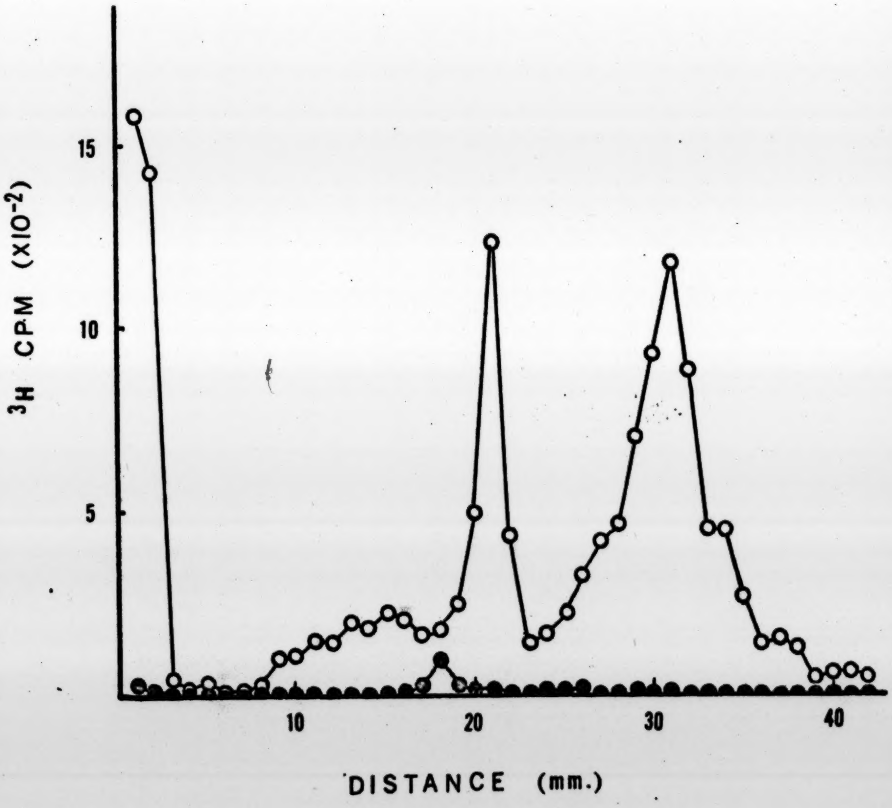

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Table II
Partition of RNA between supernatant and pellet fractions

	Total RNA by O.D. 260	Added ^{32}P-poliovirus RNA in SS region	^{3}H product RNA
Supernatant	145 μg (27%)	1350 c.p.m.(36%)	4900 c.p.m. (3.4%)
Pellet	390 μg (73%)	3750 c.p.m.(64%)	144,00 c.p.m. (96.6%)

Purified RNA from the supernatant and pellet fractions of the experiment described in Figure 15 was evaluated for total concentration by means of O.D. 260. The O.D. 260/280 ratio was 1.85 for both samples of RNA. Intact ^{32}P-labeled poliovirus RNA was determined by determining the ^{32}P c.p.m. in the SS region gels after analysis of the RNA by gel electrophoresis. ^{3}H RNA synthesized *in vitro* was determined by acid-precipitation of samples of the RNA extracted from the supernatant and pellet fractions.

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on RNA synthesis by the particulate polymerase preparation. Because these studies were concerned with total incorporation at a single time point and did not consider the individual kinetics of synthesis of RI, RF and SS RNA, incorporation studies were conducted utilizing analysis by electrophoresis of the product RNA in order to detect any subtle effects that guanidine might have in vitro. The electrophoretic profiles of RNA at two different time points were unaltered by the presence of 3mM guanidine HCl (Figure 17). These results provide further support for the view that guanidine-HCl has no effect on the activity of isolated poliovirus RNA-dependent RNA polymerase by eliminating the possibility either that the single time point selected by Baltimore failed to reveal an effect on the kinetics of the reaction,or that measurements of total incorporation of RNA precursers were failing to detect alterations in the distribution of radioactivity between RI, RF and SS species of viral RNA.

Figure 17. Effect of guanidine on the pattern of enzymatic labeling of virus-specific species of RNA. Standard reactions, one of which contained 3mM guanidine-HCl, were incubated for 15 minutes (Panel A) or 30 minutes (Panel B), and the RNA was extracted and analyzed by means of gel electrophoresis. 3mM guanidine-HCl (●- - - - -●), Control (○——○).

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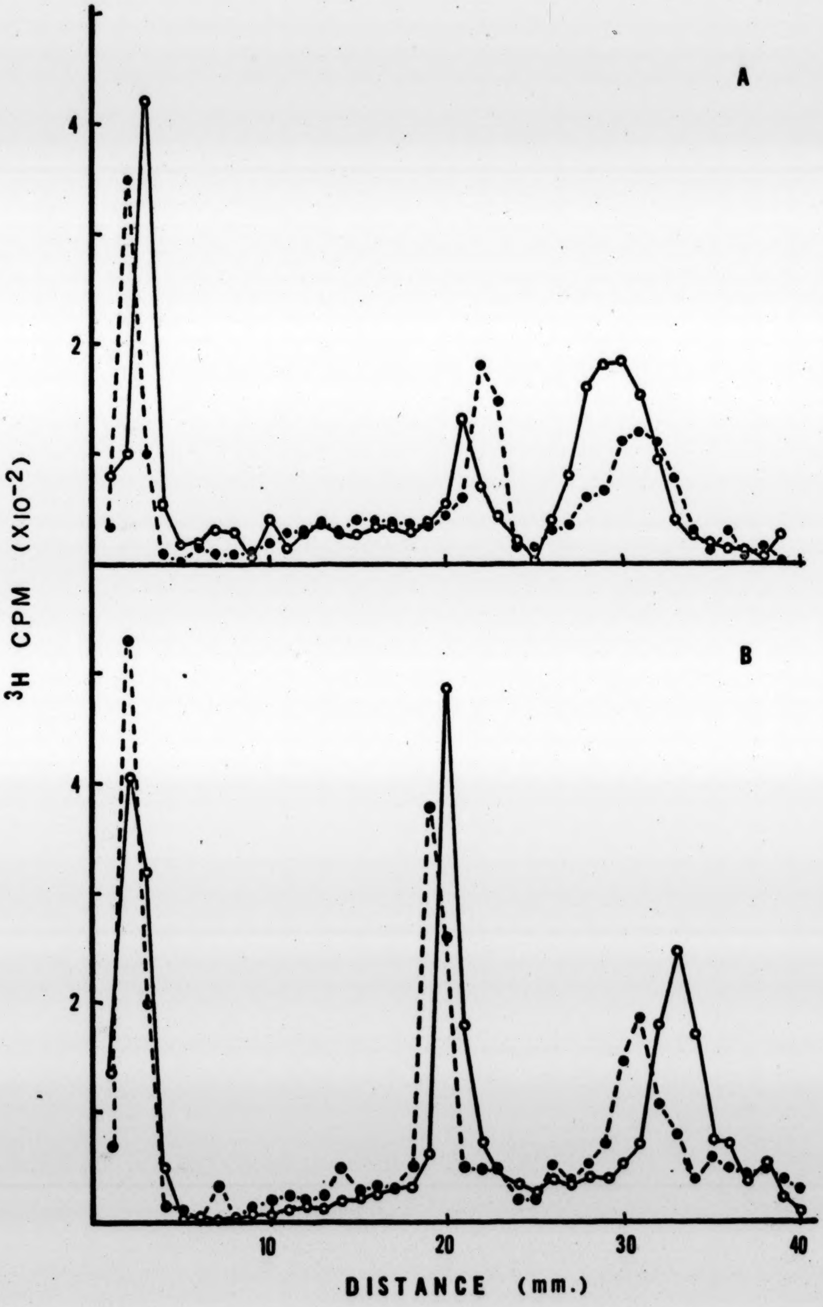

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Discussion

Biochemical studies of picornavirus-infected cells and physico-chemical and biological characterization of virus-specific RNA from these cells have provided most of the insights into how picornaviruses are replicated. The very high particle to plaque-formed unit (PFU) ratio of picornaviruses, however, has been an insurmountable obstacle to conducting experiments designed to follow the course of input viral RNA into replicating forms and possibly into progeny genomes. This obstacle is not encountered with RNA phages, and yet the most enlightening experiments concerning replication of these genomes have been conducted in vitro with purified replicase and viral RNA (Spiegelman et al., 1968). While it is a reasonable assumption that the minimal RNA viruses replicate in a manner similar in broad outline if not in detail to the RNA phages, significant differences do exist between these two groups of viruses, the most significant of which are the size of genome (Granboulan and Girard, 1969) and translational punctuation of the genetic information encoded in the genome (Baltimore et al., 1969). Thus, purification of a picornavirus RNA polymerase to the point of template dependence would provide the means for a detailed comparison of the molecular events leading from parental to progeny RNA genomes of minimal RNA viruses in prokaryotic and eukaryotic cells. Unless and until a system can be found in which the particle:PFU ratio approaches unity, or a picornavirus RNA polymerase is purified to a template-dependent state, these initial molecular replicative events will remain unresolved. The stability of this enzyme will also remain an open question because

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all measurements of activity of the crude enzyme are affected by the state of the template RNA as well as that of the enzyme itself.

In its membrane-associated state, the polymerase more than likely only adds nucleotides to pre-existing nascent RNA chains because even at optimal conditions, enzymatically synthesized RNA constitutes only 0.1 to 1% of the endogenous RNA of the enzyme preparation. This is sufficient, however, to provide unequivocal evidence that the isolated enzyme complex incorporates nucleotides into RNA having all of the physico-chemical characteristics of the major intracellular virus-specific RNA species. These studies demonstrate that the method of polyacrylamide gel electrophoresis originally designed for the study of intracellular forms of viral RNA, serves equally well for RNA synthesized *in vitro*, and provides a convenient means of precisely and simultaneously determining the progression of synthesis of these forms. Furthermore, it has been shown that the difficulties encountered with isotope dilution studies conducted *in vivo* can be circumvented with isolated RNA polymerase preparations. This experimental method results in a clean demonstration that the multi-stranded RI serves as a functional intermediate in the synthesis of single-stranded RNA, while the double-stranded RF also appears to be an end product.

The association of the functional RNA polymerase with particulate membrane structures, while representing an inconvenience in purification, may well represent a suitable model system for the elucidation of the long neglected role of membranes in viral replication. The foregoing experiments conducted with added viral RNA with and without DOC suggest that membranes play a more criti-

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cal role in viral RNA synthesis than simply serving as a delimiting structure for ribonucleases. Virus-specific membranes may be essential for the sequestration of viral RNA into a state not accessible by ribonucleases. If this be the case, then there is an additional variable to be considered in assessing the activity of RNA-dependent RNA polymerases, namely, the integrity of the membrane associated with the replicating structures. Under these circumstances, apparent inhibition of enzymatic synthesis of RNA might be indicative of a normal polymerase synthesizing a normal viral RNA product which is promptly degraded due to insufficient sequestration of the product within a virus-specific membranous body. The alteration in enzyme kinetics effected by DOC (Figure 10) could be explained by this mechanism, for example.

In an ambitious undertaking, Andre Lwoff has proposed a unifying concept to explain diverse and sometimes contradictory data concerning viral infection and host responses to this infection at the cellular as well as systemic levels (Lwoff, 1969). The central factor in this concept is the virus-mediated alteration of the stability of the host cell lysosomes, leading to the release of ribonucleases which then limit the infectious process by degrading viral RNA molecules. Moreover, factors such as lower pH and higher temperatures, which serve to disrupt lysosome membranes, also limit virus replication while cortisone, which stabilizes lysosomes, increases the severity of virus infections. The arguments could be easily shifted from host lysosomes to the stability of virus-specific membranes and better account for the data of the above experiments.

For, though the active state of the ribonucleases observed in standard enzyme preparations (Figure 14) could be explained as being due to disruption of lysosomes during the isolation procedure, the efficient protection of nascent viral RNA from these active nucleases is not in accord with Lwoff's proposal. Alternatively, the sensitivity of viral RNA to ribonucleases may be due to the integrity of virus-specific membranous components, in which case it would be these membranes rather than host cell lysosomes which would be affected by such factors as pH, temperature, and cortisone.

The role of guanidine as an antiviral agent in picornavirus infections might also be explained in terms of virus-specific membrane synthesis. Circumstantial evidence supporting this proposal includes: 1) the role of membranes in protecting virus-specific RNA from nucleases, 2) the fact that guanidine does not affect RNA synthesis in vitro in any observable way, 3) guanidine inhibition is reversed by choline and methionine (Lwoff and Lwoff, 1964; Philipson et al., 1966), metabolites known to be important in the synthesis of membrane precursors, 4) poliovirus infection at low multiplicities stimulates uptake of labeled choline into acid precipitable structures, and guanidine reverses this uptake (Penman, 1965), 5) guanidine inhibition is apparently manifested at some point in the synthesis of progeny RNA (Noble and Levintow, 1970) or its packaging into virions (Baltimore, 1968), and 6) virus infection leads to a great proliferation of membranous structures within the infected cell. The specific hypothesis would be that viral synthesis requires on-going membrane synthesis directed, at least in part, by a virus-specified enzyme. This enzyme would be susceptible to inhibition by guanidine--possibly in a competitive fashion--due to the steric and ionic resemblance of guanidine to the quaternary amine structure of choline. Inhibition of this enzyme would result in deficient synthesis of the membranes necessary for either protection of progeny RNA from nucleases or for its sequestration into a pool for packaging into progeny virions. Guanidine-dependent mutants of poliovirus would be explained by a mutant membrane synthesizing enzyme which requires the presence of guanidine in order to assume an active conformation. It is recognized that this hypothesis is highly speculative, but it has been presented in an attempt to direct attention to the role of membranous components in virus replication.

As has been previously discussed, definitive studies on the stability of RNA polymerase and on the initial events of replication of the genomes of picornaviruses must await isolation of the enzyme from ribonuclease and endogenous template RNA. Formidable obstacles, however, appear to impede such an accomplishment, at least in the immediate future. For one, there is the technological obstacle of propagating and infecting sufficient numbers of cells to obtain meaningful quantities of polymerase. Secondly, the activity that is initially assayed is not the activity which is to finally be assayed; the activity originally detected with endogenous template RNA must ultimately be detectable only in the presence of added RNA. This may require a blind purification step unless a purification procedure can be employed which serves as a transition whereby the enzyme activity is rendered partially template-dependent. Also, if the phage replicases represent the prototype of the enzymes responsible for gene duplication in picornaviruses, then only intact poliovirus RNA will be capable of directing RNA synthesis by the poliovirus RNA polymerase. Thirdly, because the viral RNA polymerase protein almost certainly represents an extremely small percentage of total host cell protein, isolation of this component from mammalian cells becomes ocrrespondingly more difficult than the isolation of replicase from bacterial cells. Fourthly, evidence is accumulating that Qβ polymerase is unique among phage replicases in its stability. Thus, it may be quite presumptuous to consider that picornavirus RNA-dependent RNA polymerases are amenable to purification analogous to that of Qβ replicase, in as much as it is becoming apparent that this purification scheme cannot even be extrapolated to the replicases of the other closely related RNA phages. And lastly, the fact that poliovirus RNA is probably translated into a single, large polypeptide which is subsequently degraded into functional virus-specific proteins (Baltimore et al., 1969) suggests that it may be biologically advantageous for the virus to synthesize a highly unstable RNA polymerase in order to avoid the complications of having one polymerase molecule for every capsid protein molecule as the replicative cycle proceeds.

If purification of a picornavirus RNA polymerase to the extent of identification of its polypeptide subunits is to be achieved, perhaps a necessary precondition would be the development of a technique making use of a mutation or metabolic effector, permitting the generation of large quantities of the RNA polymerase within the infected cells. Other likely steps may include: 1) a means of freeing the activity from membrane

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association, 2) rate-zonal sedimentation for the purpose of separating the enzyme from ribonucleases (which will presumably sediment much more slowly), 3) chromatography on phosphocellulose which will likely free the enzyme of endogenous template due to strong charge repulsion between the column medium and the nucleic acid (Zubay et al., 1971). At this point in time success of such a venture must be considered a long shot.

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Part II
The RNA and DNA-Dependent DNA Polymerases of Rous Sarcoma Virus

Introduction

Just as bacteriophages have been vital in the elucidation of the bacterial systems of macromolecular synthesis and regulation, viruses are also regarded as a means of probing the vastly more complex molecular processes of growth and differentiation in eucaryotic cells. While all virus systems characterized to date have been shown to obey the complementary base-pairing rules of Watson and Crick, and to utilize the conventional mechanisms of protein synthesis, the fact that different virus groups may possess fragmented single or double-stranded RNA genomes assures the existence of novel replicative and transcriptional processes in cells infected by these viruses. It has become clear in the last five years that an important means by which viruses accomplish these novel processes is through use of virion-associated nucleic acid polymerases which use the virus genome as template and which are activated upon entry of the virus into the host cell.

The pox viruses, which carry out their growth cycle in the cytoplasm of infected cells, and therefore presumably do not have access to host RNA polymerases, rely for synthesis of their early messenger RNA on a transcriptase function which appears to be an integral component of the nucleoprotein core (Kates and McAuslan, 1967). Reovirus, having a double-stranded RNA genome, likewise possesses a core transcriptase capable of generating its messenger RNA by a conservative mechanism of RNA synthesis (Shatkin and Sipe, 1968). Also, vescicular stomatitis virus (VSV), which

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contains one molecule of single-stranded RNA, carries with it an enzyme capable of synthesizing a series of smaller complementary RNA species which are incorporated into polysomes (Baltimore et al., 1970). More recently virion-associated RNA polymerases have been reported to be present in paramyxoviruses (Huang et al., 1971) as well as in myxoviruses (Chow and Simpson, 1971).

Perhaps the most remarkable of the virion-associated nucleic acid polymerases, however, is that which was discovered independently by Temin and Mizutani (1970) and by Baltimore (1970) to be present in RNA tumor virus preparations. Howard Temin had long before predicted a catalytic step in the infectious cycle of RNA tumor viruses involving the synthesis of a DNA complement of the RNA genome of the virus (Temin, 1964a). The experimental basis for this hypothesis was provided primarily by the effects of inhibitors of DNA synthesis and DNA-dependent RNA synthesis on the growth cycle of RNA tumor viruses. Temin (1963) demonstrated that virus production can be interrupted at all times by actinomycin D, while Bader (1964) and Temin (1964b) showed that inhibition of DNA synthesis soon after infection also resulted in the inhibition of virus production. Although the significance of these early studies with inhibitors of DNA synthesis was severely undermined by later findings which suggested that their mode of action may simply be to block the cell devision necessary for the establishment of infection, better evidence was provided that DNA synthesis other than that of cellular S phase, is required for the establishment of infection by the RNA tumor viruses (Temin, 1967). It was shown that partially synchronized secondary culture cells in G_2 can be infected with RSV and then treated for 12 hours with

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inhibitors of DNA synthesis, after which time virus production is completely blocked while cell growth proceeds normally. Thus, Temin, armed with this experimental evidence, the precedent for nucleic acid polymerases associated with the virions of two widely differing groups of animal viruses, and an abiding conviction that the genomes of RNA tumor viruses utilize DNA as the minus strand for the synthesis of progeny RNA genomes, undertook a search for an RNA-dependent DNA polymerase in the virions of such viruses.

David Baltimore initiated a similar search utilizing a quite different rationale. The initial factor in the evolution of this rationale was the discovery that picornavirus infection results in the synthesis of large polypeptides which are subsequently degraded into the virus-specific capsid and non-capsid proteins (Maizel and Summers, 1968; Jacobson and Baltimore, 1968). From this, Baltimore surmised that animal cells must lack the means for termination and reinitiation of synthesis (internal initiation) of polypeptides on polycistronic m-RNA molecules. Thus, the RNA viruses of animals would be confronted with an obstacle not presented to the RNA bacteriophages--namely, that polycistronic messages cannot be employed for the direct synthesis of multiple polypeptides. Accordingly, the polycistronic messages of the picornaviruses would, of necessity, be translated into large precursor polypeptides which would generate biologically useful capsid and non-capsid proteins only upon being fragmented by specific protolytic cleavage. Alternatively, the RNA genomes of other viruses such as the reoviruses and myxoviruses might bypass this obstacle by possessing fragmented genomes which can presum-

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ably generate monocistronic messages. A further contribution to this rationale was provided when Baltimore et al.(1970) discovered within virions of VSV, an RNA polymerase which generates smaller (-) strand messenger RNA molecules from a single, large (+) strand RNA genome. This was interpreted as yet another means by which a virus might overcome the "punctuation" problem. Baltimore reasoned from all of this that the RNA tumor viruses might indeed transcribe their genomes into DNA as proposed by Temin, for this DNA could then serve in a conventional fashion to transcribe appropriate messenger RNA species, and in so doing, circumvent the host cell translation deficiencies. Thus, Baltimore (1970) and Temin and Mizutani (1970), employing quite different rationales, came to look for and to find RNA-directed DNA polymerase activities in preparations of murine leukemia and avian sarcoma viruses.

The research to be discussed here was undertaken soon after this discovery with the intention of characterizing the RSV-associated polymerase(s) and their catalytic potential in order that such information might serve in the elucidation of the biochemical mechanisms by which the RNA tumor viruses replicate and bring about cellular transformation. Much of this work has been published (Garapin et al., 1970; McDonnell et al., 1970 and 1971).

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Materials and Methods

Preparation of virus. The purified Schmidt-Ruppin strain of RSV used in these investigations was provided by Dr. Warren Levinson and Dr. J. Michael Bishop. The propagation and purification of this virus has been previously described (Bishop et al., 1970a).

Conditions for assay of the RSV DNA polymerase. The standard assay mixture contained: 0.1M Tris-HCl, pH 8.1; 0.01M $MgCl_2$; 0.08mM dATP, dCTP, and dGTP; 2.0% (v/v) β-mercaptoethanol; 0.8μM ^3H TTP (11 Ci/mM). For assay of the virion-associated DNA polymerase activity, an appropriate concentration of NP_{40} was added to the assay mixture. The detergent concentration was determined independently for each virus preparation, and usually varied between 0.05 and 0.1% NP_{40} (v/v). Detergent was not required for assay of the isolated DNA polymerase. Incubation was at 37°. Samples for the determination of total acid-precipitable radioactivity were added to 0.5 ml of 0.1M sodium pyrophosphate and precipitated by the addition of 2.0 ml of 3.5% perchloric acid containing 0.1M sodium pyrophosphate. After 10 minutes at 0°, the precipitates were collected on glass filters, washed seven times with 0.1N HCl in 0.1M sodium pyrophosphate, and once with 95% ethanol. The filters were dried and counted in 5.0 ml of 4.2% (v/v) liquifluor in toluene at ambient temperature in a Beckman liquid scintillation spectrometer. The complete assay mixture was prepared by diluting the enzyme preparation into 2.5 parts of a solution made up of the three unlabeled nucleoside triphosphates at 1.6μM, 20mM $MgCl_2$,

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and 4% (v/v) β-mercaptoethanol in 0.1M Tris-HCl, pH 8.1; ½ volume of 8μM ^{3}H TTP in 0.1M Tris-HCl, pH 8.1; and 1.5 volumes of 0.1M Tris-HCl, pH 8.1. In assays utilizing non-ionic detergent, the appropriate volume of NP_{40} was added and this volume was substrated from that of the Tris buffer.

Conditions for assay of the DNA-dependent DNA polymerase. Conditions were the same as above except that the final addition of 0.1M Tris-HCl was reduced to 1 volume and ½ volume of calf thymus DNA at 2 mg/ml in 0.1M Tris-HCl, pH 8.1 was added, resulting in a final concentration of DNA of 200 μg/ml.

Preparation of DNA. The calf thymus DNA was prepared by dissolving spooled (Sigma) DNA at 10 mg/ml in 0.1M Tris-HCl, pH 8.1 for two days at room temperature. The solution was cooled to 0° and sonicated for 6 minutes with a Branson 75 Sonicator at power setting 3. In order to avoid overheating of the solution, the sonication schedule was carried out at 1 minute periods of sonication alternating with 1 minute intervals of cooling.

Preparation of single-stranded DNA. Sonicated calf-thymus DNA prepared as described above was denatured by addition of 0.25 volumes of 2.0N NaOH. After 15 minutes at 0° the solution was neutralized with 2.0N HCl. This solution was added to hydroxyapatite in 0.01M $NaPO_4$ buffer, pH 6.8 at a ratio of 1 mg DNA/ml of packed hydroxyapatite. DNA eluted from the hydroxyapatite between 0.05M and 0.15M phosphate buffer at 56° was taken as single-stranded calf thymus DNA. This fraction represented ca. 40% of the input DNA, and displayed no increase in

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O.D.$_{260}$ upon subsequent denaturation in alkali.

Extraction of nucleic acids. Nucleic acids were extracted from reaction mixtures by treating these mixtures with 0.5% (w/v/) sodium dodecyl sulfate and self-digested pronase (0.5 mg/ml) at 37° for 45 minutes. For some analyses this treated sample was used directly, but for others the nucleic acids were further purified by two extractions in an equal volume of phenol at room temperature and precipitation in 2 volumes of absolute ethanol at -20°.

Reagents. The sources of reagents were: Deoxyribonucleoside triphosphates, dATP, dCTP, dGTP and TTP (Calbiochem.); ^{3}H TTP, 10-15 Ci/mM (New England Nuclear Corp.); ^{32}P dGTP (International Chemical and Nuclear Corp.); E. coli, alkaline phosphatase and pancreatic ribonuclease A (Worthington Biochemicals, Inc.). Stock solutions of RNase at 10 mg/ml were boiled for 10 minutes in order to inactivate enzyme contaminants (i.e. DNase), pronase, B grade (Calbiochem.). Stock solutions at 10 mg/ml were self-digested at 37° for 2 hours in order to degrade protein contaminants (i.e. RNase and DNase); dodecyl sodium sulfate (SDS) (Matheson, Coleman and Bell); non-idet P$_{40}$ (NP$_{40}$) (Shell Chemical Co.); phenol, reagent grades and toluene, analytical grade (Mallinckrodt); Liquifluor (New England Nuclear Corp.); cesium sulfate, optical grade (Gallard Schlesinger); hydroxyapatite, "Bio-Gel HT," (Bio-Rad Laboratories) actinomycin D was a gift from Merck, Sharp and Dohme, Inc.; ^{32}P-labeled and unlabeled phage lambda DNA was generously provided by Dr. H. Boyer.

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Results

pH ionic and temperature requirements of virus-associated RNA dependent DNA polymerase. The reaction mixture originally devised in this laboratory to assay the DNA polymerase of non-ionic detergent disrupted RSV virions required Tris buffer at pH 8.7 in combination with phosphoenolopyruvate (PEP) and pyruvate kinase. This requirement for a triphosphate generating system has not been adequately explained, but it may have been due in part to the fact that the PEP and pyruvate kinase were not themselves adequately buffered, and in combination, were capable of lowering the pH of the buffered reaction by about 1 pH unit to ca. pH 7.7. (Due to the small volumes of these reaction mixtures, pH determinations were made with pH paper and were subject to the limitations of accuracy of this method). In subsequent assays conducted without the triphosphate generating system, the final pH of the reaction was essentially that of the Tris buffer. Incorporation is maximal at pH 8.1 and above, but falls to 50% at pH 7.2 (Figure 1A). Monovalent cations were also tested for their ability to stimulate activity, but all were found to inhibit incorporation at concentrations above .02M salt, whether it be NaCl, KCl, or NH_4Cl (Figure 1B). The effects of temperature on catalysis were investigated for two reasons. First, it was surmised that the enzyme would most likely be maximally active at 42°, the temperature of the host organism. Secondly, the "core"-associated RNA polymerase of reovirus displays remarkable increases in activity at elevated temperatures. As expected, the enzymatic activity proved to be

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Figure 1. Properties of the RSV virion-associated DNA polymerase.

Panel A--Enzyme activity as a function of pH. Reactions of 0.1 ml were incubated for two hours at 37° in conditions similar to the standard assay mixture except that the pH of the Tris buffer was varied as indicated.

Panel B--Enzyme activity as a function of monovalent salt concentration. Reactions of 0.05 ml containing 5.0 ml of purified RSV were assayed for 2 hours at 37° in the presence of various concentrations of NaCl, KCl, or NH_4CL. The plot of relative activity as a function of salt concentration is applicable for each of the monovalent cations.

Panel C--Effect of elevated temperature on enzyme kinetics. Standard reaction mixtures were assayed at either 37° or 42° and samples for the determination of acid-precipitable radioactivity were withdrawn at the indicated times. 37°(○——○——○); 42°(●- - -●- - -●).

Panel D--Enzyme activity as a function of temperature. Standard reaction mixtures were incubated at the indicated temperatures for either one (○——○——○) or three (●- - -●- - -●) hours, and the relative ^{3}H TMP incorporation versus temperature at these two lengths of assay was determined.

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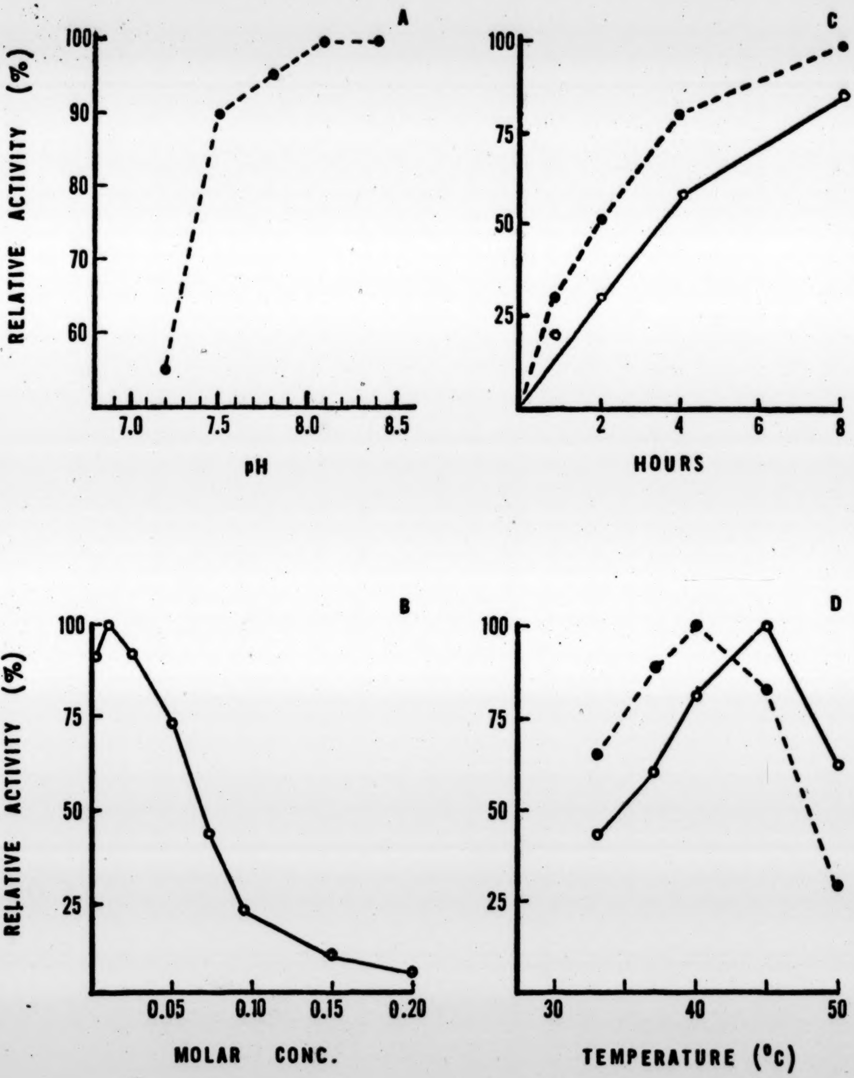
A
RELATIVE ACTIVITY (%)
100
90
80
70
60
7.0
7.5
8.0
8.5
pH
C
100
75
50
25
2
4
6
8
HOURS
B
RELATIVE ACTIVITY (%)
100
75
50
25
0.05
0.10
0.15
0.20
MOLAR CONC.
D
100
75
50
25
30
40
50
TEMPERATURE (°c)

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as stable at 42° as at 37°, and consequently the rate of synthesis was somewhat greater. At yet higher temperature however, concomitant increases in the initial rates of synthesis are ever more quickly counterbalanced by decay of the enzymatic activity (Figure 1D). A temperature of 37° was decided upon as the standard assay temperature, primarily for purposes of convenience.

Delineation of two enzymatic activities. If the presence of this enzyme was to be biologically significant, it was reasoned that at least two enzymatic steps must be involved. The first reaction would be expected to utilize the genome RNA as template for the synthesis of an RNA:DNA hybrid, and the second reaction would employ the DNA of this hybrid as template for the synthesis of double-stranded DNA. Actinomycin at 50 μg/ml was found to reduce the activity of the RSV DNA polymerase by ca. 50% (McDonnell et al., 1970), so it became of interest to determine if that enzymatic activity which requires DNA as template is selectively inhibited, as would be predicted from binding characteristics of actinomycin D. Reactions with and without actinomycin D were incubated for 4 hours after which the nucleic acids were extracted and analyzed by isopycnic centrifugation in gradients of Cs_2SO_4 (Figure 2). While the normal 4 hour product contains DNA distributed in two populations--the heavier corresponding to DNA in association with larger RNA molecules and the lighter similar in density to single or double-stranded DNA--the DNA synthesized in the presence of actinomycin D lacks the component which bands with the lambda DNA marker. It is postulated from this that actinomycin D does

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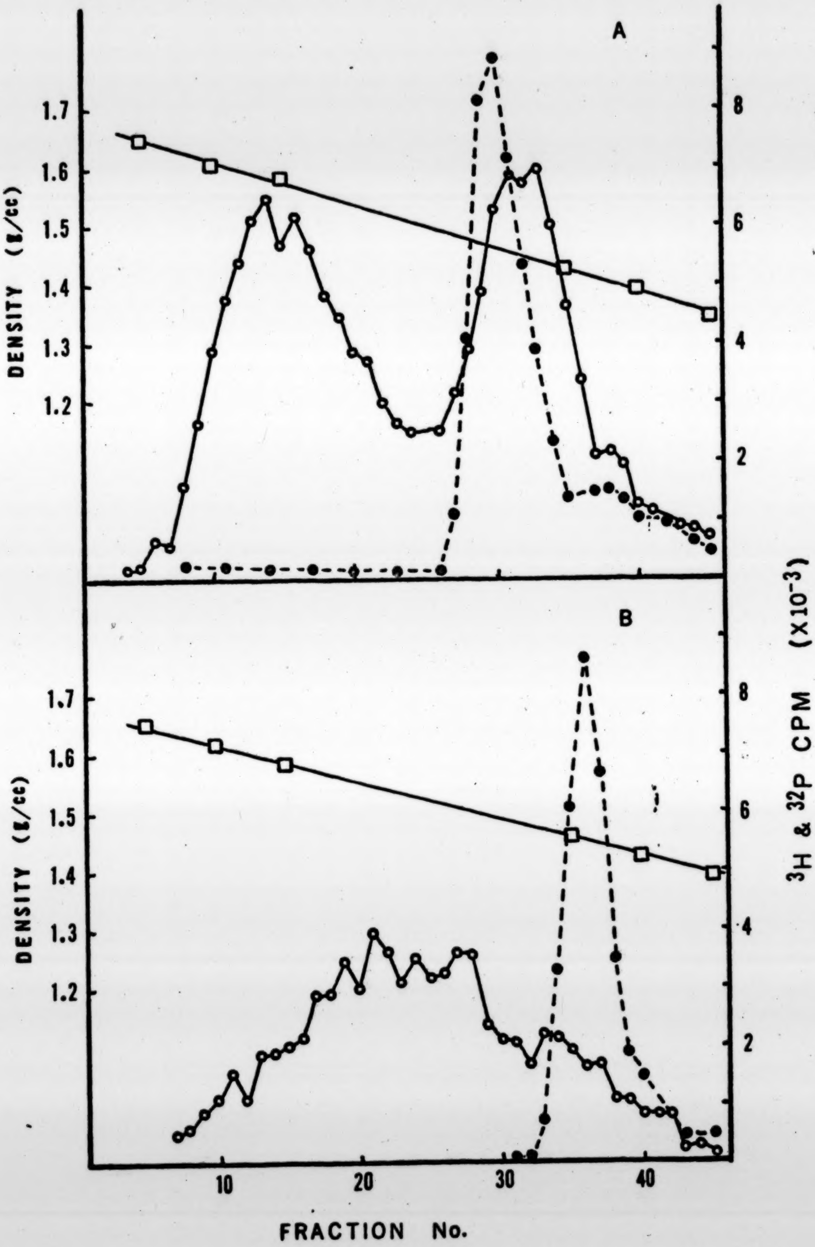
Figure 2. Isopycnic centrifugation of the enzymatic product. The nucleic acid products from standard and actinomycin D treated standard reactions were extracted as described in Materials and Methods, and dialyzed overnight against 0.1M NaCl-0.002M EDTA-0.02M Tris-HCl, pH 7.4. The volumes of the samples were then adjusted to 3.0 ml with 0.002M EDTA and 0.02M Tris-HCl, pH 7.4. Crystaline $CsSO_4$ was added to a final density of 1.52 g/cc as determined by refractive index; the samples were placed in polyallomer tubes and mineral oil was layered over the samples to within 2 mm of the top. The tubes were centrifuged at 33,000 r.p.m. in an SW-50 rotor, for 60 hours at 4°. Five drop fractions of the gradients were collected, and the radioactivity of each was determined by acid-precipitation. The densities of several samples were also determined by measurement of the refractive index.

Panel A--Product of control reaction incubated 4 hours with ^{32}P phage lambda DNA denatured in alkali as marker.

Panel B--Product of reaction containing actinomycin D at 50 g/ml with native ^{32}P lambda phage DNA as marker. ^{3}H C.P.M. (○———○); ^{32}P C.P.M. (●- - -●); buoyant density (g/cc) (□———□).


A
B
DENSITY (g/cc)
1.7
1.6
1.5
1.4
1.3
1.2
8
6
4
2
^{3}H & ^{32}P CPM (X10^{-3})
10
20
30
40
FRACTION No.

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not interfere with synthesis of RNA:DNA hybrid molecules, but that it blocks synthesis of double-stranded DNA, presumably by binding to either a hybrid or single-stranded DNA precursor.

Stimulation of DNA synthesis by exogenous DNA. The foregoing results suggested the possibility that exogenous DNA might be capable of serving in place of the nascent DNA as template for double-stranded DNA synthesis. In order to test for this possibility, detergent disrupted RSV was first pre-incubated with 1 mg/ml ribonuclease to minimize DNA synthesis on endogenous RNA, after which the polymerase assay was conducted in the presence of sonicated calf thymus DNA. DNA synthesis in these conditions was not only detected, but occurred at an even greater rate than that of the unmodified reaction (Figure 3A). In more recent experiments, this difference has been shown to be even more dramatic (Figure 4B). That this activity is analogous to the actinomycin-sensitive activity in virions is demonstrated in Figure 3B. Actinomycin D at concentrations analogous to those of Figure 2 reduces the exogenous DNA stimulated DNA polymerase activity by more than 50%, even at the very high concentrations of DNA used as template for this reaction. Thus, actinomycin D does have a direct effect on the second, or DNA-dependent activity, even when this activity is directed by exogenous DNA.

Association of DNA-dependent DNA polymerase with RSV virions. The DNA polymerase activity responsive to exogenous DNA was shown to be intimately associated with virions simply by the demonstration that this activity is completely dependent on the presence of non-ionic detergent (Figure 4A). If 0.1% NP_{40} is added at 30

Figure 3. Response of RSV-associated DNA polymerase to calf thymus DNA. Replicate reaction mixtures containing 10μg of RSV protein (10μl of purified RSV) plus 30μl of a complete mixture containing 0.1% NP_{40} but lacking ^3H TTP were modified by adding 5μl of either a) pancreatic RNase at 10 mg/ml in water, b) water, c) 0.5 mg/ml actinomycin D in 50% ethanol, or d) 50% ethanol. All samples were preincubated for 15 minutes at 0° after which 5μl of ^3H TTP and 5μl of calf thymus DNA at 2.0 mg/ml in 0.1M Tris-HCl, pH 8.1, or 5μl of the Tris buffer were added. Incubation was at 37° and 10μl withdrawals were made at the indicated times.

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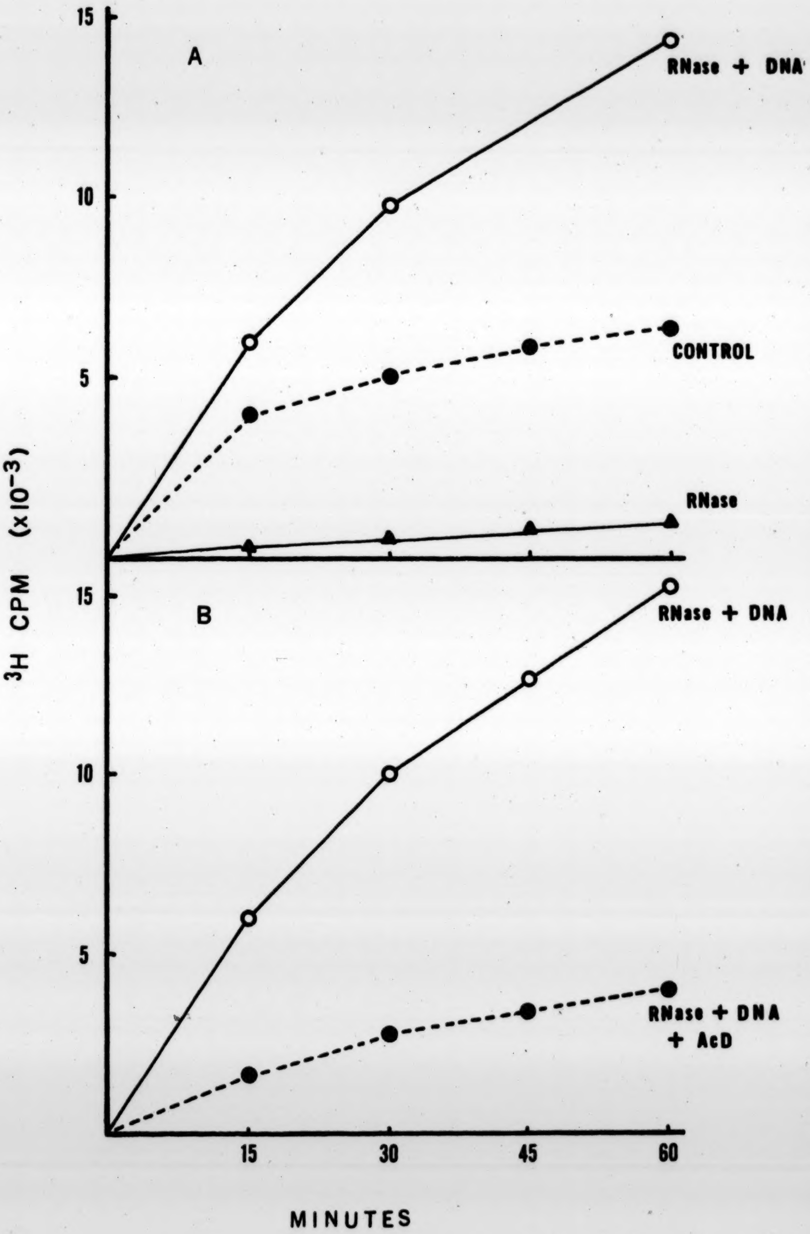
A
B
15
10
5
15
10
5
^{3}H CPM (x10^{-3})
RNase + DNA
CONTROL
RNase
RNase + DNA
RNase + DNA
+ AcD
15
30
45
60
MINUTES

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Figure 4. Detergent requirement for exogenous DNA-dependent DNA synthesis.

Panel A--Reaction mixtures similar to those of Figure 3 containing RNase and DNA but lacking NP$_{40}$ were assayed as in Figure 3, and compared with a control containing 0.1% NP$_{40}$. Control (○——○——○); Minus NP$_{40}$ (△——△——△); 0.1% NP$_{40}$ added at 30 minutes of incubation (▲- - -▲- - -▲).

Panel B--Endogenous RNA-dependent and exogenous DNA-dependent activities as a function of detergent concentration. Standard reaction mixtures of 50 μl containing the indicated concentrations of NP$_{40}$ and 10 μg of RSV protein (○——○——○) or 1 μg RSV protein and 200 μg/ml DNA (●- - -●- - -●) were incubated for 2 hours at 37°, after which acid-precipitable radioactivity was determined.

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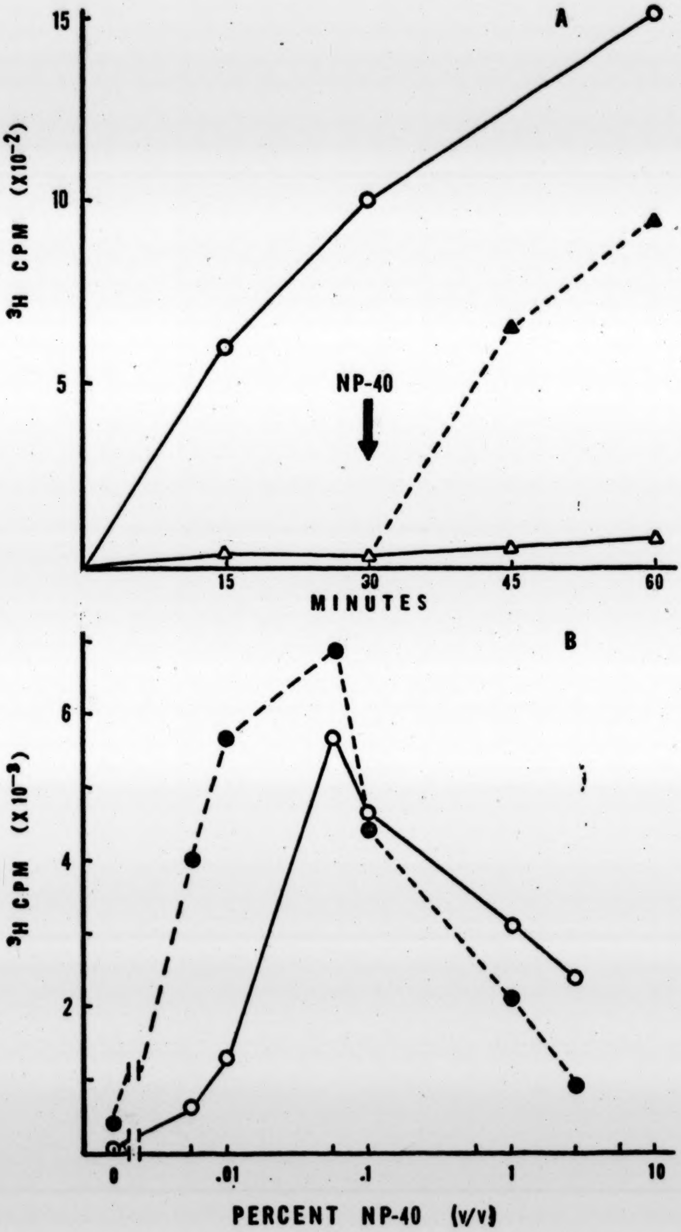
A
15
10
5
^{3}H CPM (X10^{-2})
NP-40
15
30
45
60
MINUTES
B
6
4
2
^{3}H CPM (X10^{-3})
0
.01
.1
1
10
PERCENT NP-40 (v/v)

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minutes of incubation, incorporation of deoxyribonucleotides commences at a rate comparable to the initial kinetics of a reaction containing non-ionic detergent from zero time. Also, virus preparations have been repeatedly tested for endogenous and DNA-dependent activities as a function of detergent concentration. Although there have been occasional variations, the assays containing calf thymus DNA generally have a ten-fold greater rate of incorporation and have a detergent optimum similar to that of the endogenous RNA dependent activity.

In order to avoid isotope depletion effects, the DNA-dependent detergent curve was usually conducted by utilizing 10-fold less virus (Figure 4B). Under these circumstances the DNA-dependent activity is uncovered at lower detergent concentrations than is the endogenous activity, a finding which may be accounted for by the fact that the detergent requirement varies directly with the virus protein concentration (Garapin et al., 1970).

Template requirements of the DNA-dependent DNA polymerase. Calf thymus DNA is effective in stimulating DNA synthesis before and after it has been sheared by sonication (Figure 5A). The DNA used in templating experiments is routinely sonicated for 6 minutes however, in order to reduce the viscosity for convenience of handling. If the sheared calf thymus DNA is first denatured at high pH, and the single-stranded DNA is recovered from hydroxyapatite by sodium phosphate elution (Fanshier et al., 1971), this single-stranded DNA is at least as effective per weight in stimulating DNA synthesis, as is the double-stranded DNA (Figure 5B). The sheared calf thymus DNA saturates the virion-associated

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Figure 5. Properties of the DNA polymerase activity responsive to calf thymus DNA.

Panel A--Sonication of DNA. Standard reactions of 50 μl, modified by the addition of 4 M ^{3}H TTP (5X), were incubated for 2 hours at 37° in the presence of calf thymus DNA at 2.0 μg/ml which had been sonicated as described in Materials and Methods for the indicated time periods.

Panel B--Response of DNA polymerase to single- versus double-stranded DNA. Complete standard reactions of 50 μl containing RNase at 1.0 mg/ml 10 μg RSV protein were incubated in the presence of sonicated native calf thymus DNA at 200 μg/ml (●---●---●); or in the presence of single-stranded calf thymus DNA at 200 μg/ml (○—○—○) (see Materials and Methods for preparation). Duplicate 5 μl withdrawals were taken at the indicated incubation times.

Panel C--Relative activity as a function of DNA concentration. Standard reaction mixtures of 100 μl containing 1.0 mg/ml RNase and the indicated concentrations of sonicated calf thymus DNA were incubated for 2 hours at 37°.

Panel D--DNA-dependent activity as a function of monovalent cation concentration. DNA at saturating and limiting concentrations was assayed in 50 μl reactions for its ability to stimulate DNA synthesis at the indicated concentrations of monovalent salts for 2 hours at 37°. The curves plotted were determined in the presence of NaCl; but those determined for KCl or NH_4Cl were virtually identical. DNA at 200 μg/ml (○———○); DNA at 2.0 μg/ml (●-----●).

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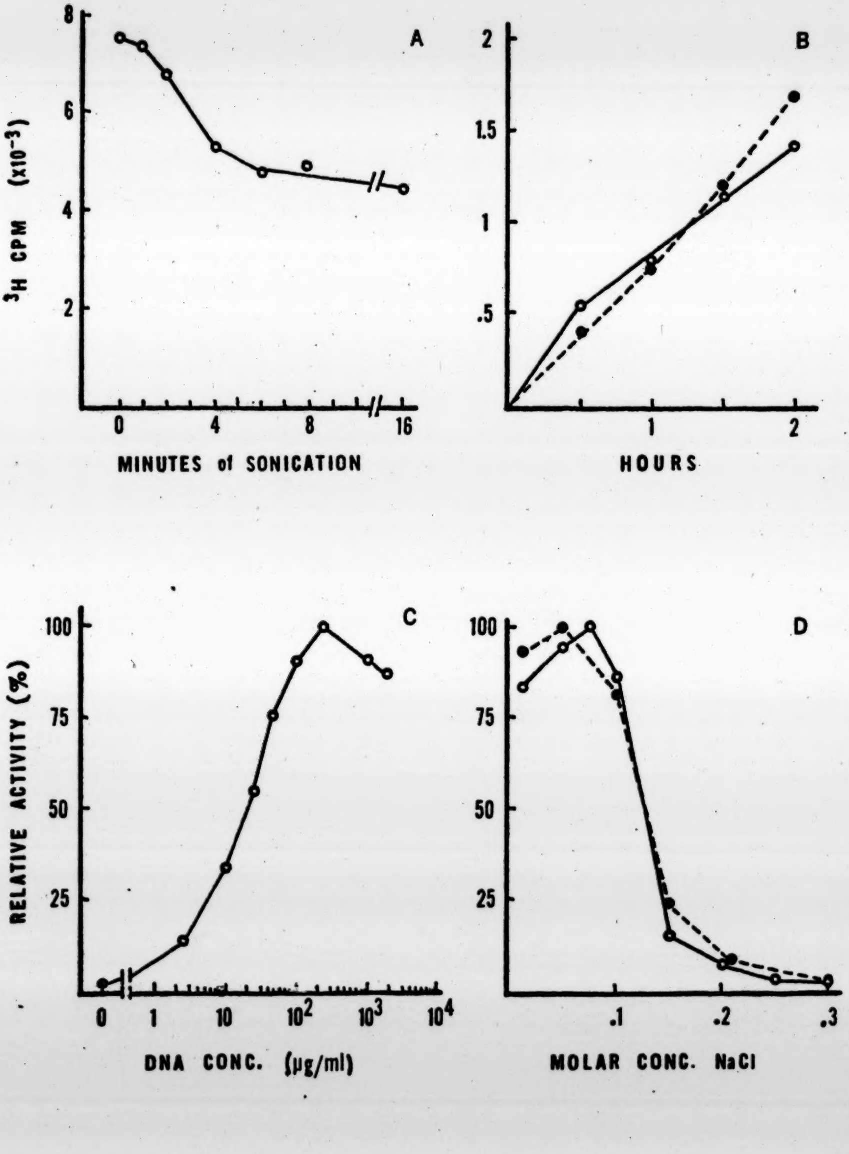
A
B
C
D
^{3}H CPM (x10^{-3})
8
6
4
2
0
4
8
16
MINUTES of SONICATION
2
1.5
1
.5
1
2
HOURS
RELATIVE ACTIVITY (%)
100
75
50
25
0
1
10
10^2
10^3
10^4
DNA CONC. (µg/ml)
100
75
50
25
.1
.2
.3
MOLAR CONC. NaCl

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DNA polymerase at approximately 250 μg/ml (Figure 5C), and this activity is also sensitive to monovalent cations, but at a substantially higher salt concentration than the endogenous RNA-dependent activity (Figure 5D). This disparity in salt sensitivity between the RNA- and DNA-dependent activities cannot simply be explained by the relative concentrations of these templates because the reaction is no more sensitive to salt when calf thymus DNA is present at 2 μg/ml than when it is present at 200 μg/ml (Figure 5D). It is rather more likely that the differing sensitivities to salt are due to unique properties of the DNA and RNA binding sites, or to differing affinities for monovalent cations between deoxyribo- and ribonucleic acids as suggested by the divergent relative densities of these molecules in cesium salts.

Solubilization of the RSV DNA polymerase. The nucleic acid polymerases associated with the cores of poxviruses and reoviruses have been refractory to all procedures designed to dissociate them from nucleoprotein complexes and/or to render them dependent on exogenous nucleic acids for activity (B. Woodson, personal communication; Joklik et al., 1970). The propensity of the DNA-dependent DNA polymerase of RSV to utilize exogenous DNA to direct DNA synthesis suggested that this enzyme might prove to be unlike previously studied virion-associated polymerases in that it well might not be firmly bound to a nucleoprotein core structure. To test for this possibility, detergent-disrupted RSV preparation was sedimented in gradients of sucrose in conditions which are normally used for the isopycnic banding of

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intact RSV virions, after which the gradient was assayed for both endogenous RNA-directed and exogenous DNA-dependent DNA synthetic activities. As can be seen in Figure 6 both activities were released from association with intact virions and sedimented at a rate characteristic of typical monomeric or oligomeric proteins. Glycerol was subsequently used in place of sucrose for the establishment of gradients for rate-zonal sedimentation analyses because of its generally superior stabilizing effects on enzymes (Burgess, 1969). The exogenous DNA-directed activity was delineated in gradients of glycerol containing 0.2M KCl as a homogenous peak with a consistent and predictable rate of migration (Figure 7), while endogenous activity was not readily detectable in these conditions.

Ammonium sulfate fractionation. As an additional purification step, ammonium sulfate fractionation of detergent-disrupted purified RSV was carried out. The endogenous and DNA-dependent activities were both found to be precipitated in 31% ammonium sulfate (Table I), a result which provides a convenient means of concentrating the enzyme from virus preparations prior to its being layered on gradients of glycerol. The precipitation of the enzyme activities may be due in part to co-precipitation of the detergent in ammonium sulfate. The precipitates were formed as a layer which was partially adherent to the sides of the tubes and partially floating on the surface of the solution. Also, by qualitative surface tension measurements, it appeared that the pattern of precipitation of the detergent in varying ammonium sulfate concentrations was similar to that of the enzymatic

Table I
Ammonium Sulfate Fractionation

Sample	$(NH_4)_2SO_4$ (M)	$(NH_4)_2SO_4$ (w/v)	Activity relative to control Supernatant	 Precipitate
1	0.6	14%	.93	.01
2	0.8	19%	.66	.11
3	1.0	23%	.50	.30
4	1.2	27%	.30	.58
5	1.4	31%	.01	.92
6	2.0	42%	.01	.82

600 μg of purified RSV protein was made 1% with respect to NP$_{40}$ in a final volume of 0.6 ml and allowed to sit for 30 minutes at 0°. The contents were divided into six aliquots of 0.1 ml and were made from 0.6 to 2.0 molar in 1.0 ml by the addition of the appropriate volumes of water and 2.0M ammonium sulfate, buffered with 0.05M Tris, pH 8.1 (4.0M ammonium sulfate was added in place of water to sample #6). Precipitates were allowed to form for 30 minutes at 0° after which the samples were centrifuged at 30,000 r.p.m. for 15 minutes at 4° in a type 40 rotor. The supernatants were collected and the precipitates were resuspended in 1.0 ml, 0.1M NaCl-0.002M EDTA-0.02M Tris-HCl, pH 7.4 (dialysis buffer). All samples were then dialyzed overnight against the dialysis buffer. 50 μl portions of each sample were then assayed for DNA-dependent activity in standard reaction mixtures for 2 hours at 37°. 5 μl samples of unfractionated purified RSV were added to 45 μl of dialysis buffer and assayed as controls. These samples gave an average of 51,600 c.p.m. in a 2 hour assay.

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Figure 6. Distribution of DNA polymerase activity upon sedimentation of detergent disrupted RSV in sucrose. 20μl of purified RSV (20μgRSV protein) was disrupted in 2.0% NP_{40} and pre-incubated for 15 minutes at 37° in 80μl of a standard reaction mixture lacking ^{3}H TTP. The sample was then layered on a pre-formed gradient of 25 to 65% sucrose in 0.1M NaCl, 0.002M EDTA, and 0.02M Tris-HCl, pH 7.4. The sample was centrifuged in an SW-50 rotor at 33,000 r.p.m. for 12 hours at 4°, after which 75μl fractions were collected.

Panel A--Endogenous activity was assayed by withdrawing 10μl samples from the even-numbered fractions and adding them to 40μl standard reaction mixtures. Incubation was for 2 hours at 37°, after which acid-precipitable radioactivity was determined. The arrow indicates the density of the gradient where intact RSV would come to equilibrium.

Panel B--DNA-dependent activity was assayed by withdrawing 10μl samples from the odd-numbered fractions and adding them to 40μl standard reaction mixtures containing 1.0 mg/ml RNase and 200μg/ml calf thymus DNA. Incubation was as above. The arrow indicates density at which intact virions would be expect to band.

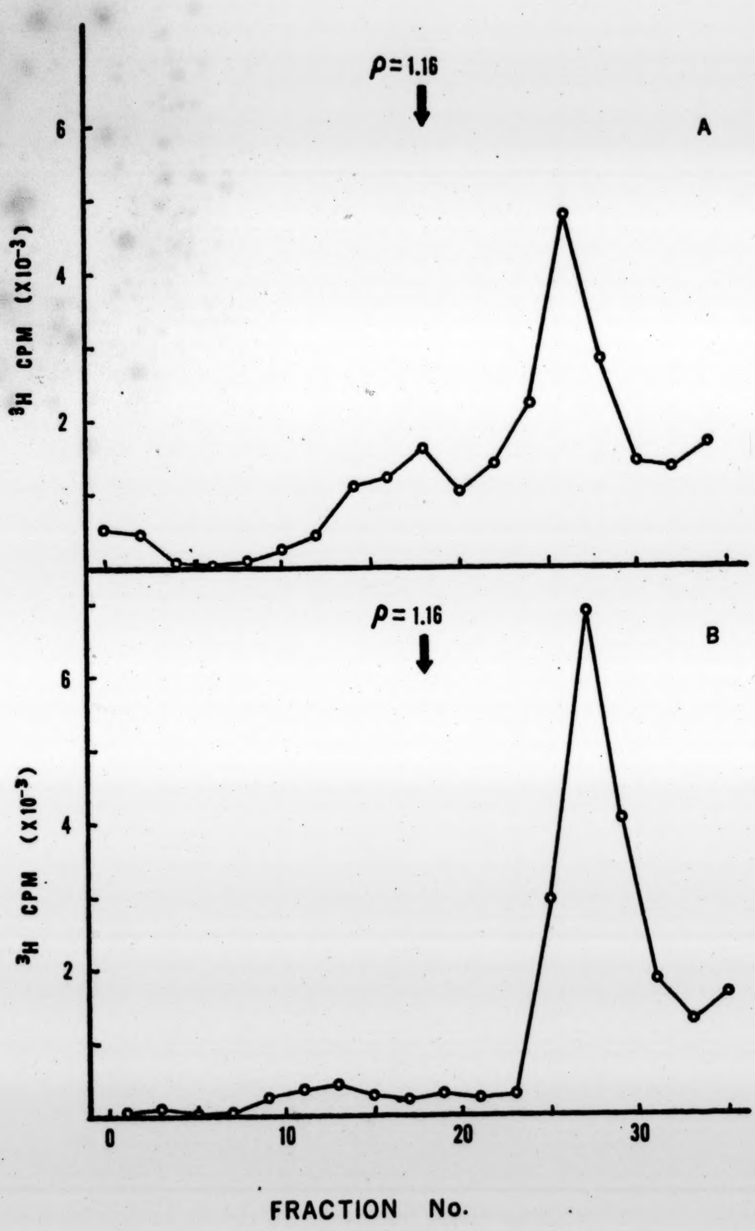

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Figure 7. Rate-zonal sedimentation of DNA-dependent DNA polymerase in a glycerol gradient.

10μl of purified RSV (18μg protein) was disrupted in 2.0% NP$_{40}$ for 30 minutes at 0°, after which 0.1 ml of 0.1M NaCl-0.002M EDTA-0.02M Tris-HCl, pH 7.4- 2% (v/v)β-mercaptoethanol was added. The sample was layered on a preformed 5.0 ml gradient of 10 to 30% glycerol (v/v) in 0.05M Tris-HCl, pH 8.1-0.2M KCl-1.0mM EDTA-2% (v/v) β - mercaptoethanol. The sample was centrifuged in an SW-65 rotor at 64,000 r.p.m. for 4 hours at 4°. After centrifugation the bottom of the polyallomer tube was punctured and 12 drop fractions were collected. For assay of endogenous activity 20μl samples of each fraction were added to 20μl of a solution containing the three unlabeled deoxynucleoside triphosphates at 0.16mM, MgCl$_2$ at 0.02M, and ^{3}H TTP at 8 M (10X). For assay of DNA-dependent activity 20μl samples of each fraction were added to 20μl of a solution containing the unlabeled triphosphates at 0.16mM, MgCl$_2$ at 0.02M, ^{3}H TTP at 1.6μM(2X), and sonicated calf thymus DNA at 400μg/ml. Assay was for 2 hours at 37°.

Endogenous activity assayed with 5X ^{3}H TTP (○——○——○); DNA-dependent activity (●---●---●).

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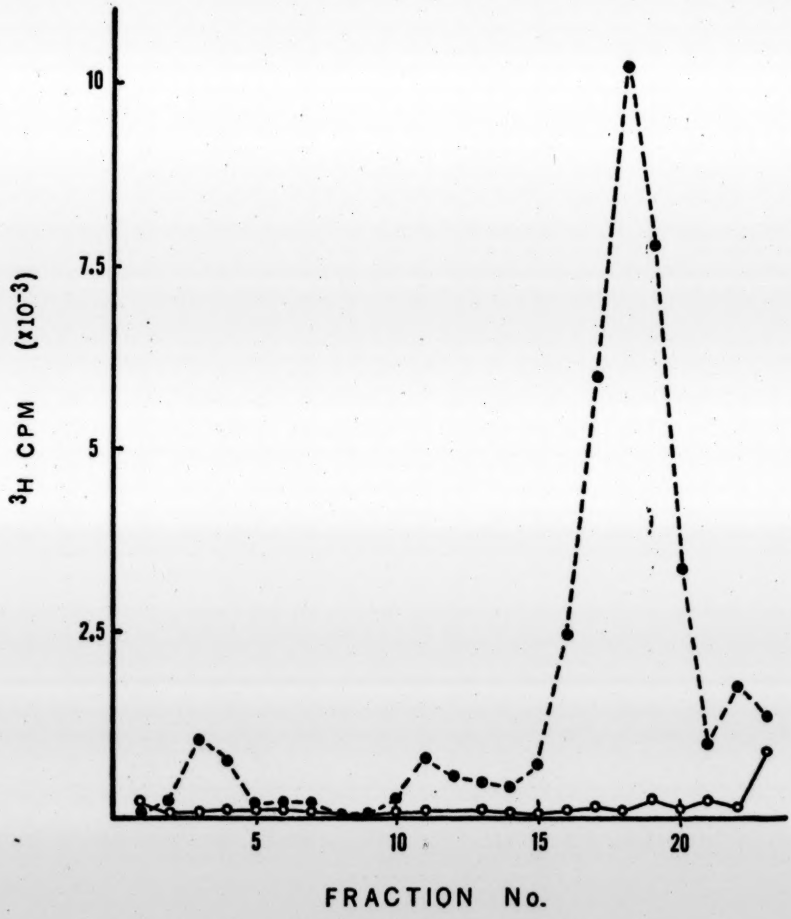

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activities.

Determination of the sedimentation coefficient of the isolated DNA polymerase. *E. coli* DNA-dependent RNA polymerase was originally selected as a sedimentation marker in order to obtain an initial estimate of the size of this enzyme. As can be seen in Figure 8A, *E. coli* RNA polymerase sediments considerably more rapidly than the RSV DNA polymerase, a result which established the soluble nature of the RSV enzyme and provided an initial estimate of ca. 7S for its sedimentation coefficient. Bacterial alkaline phosphatase is a more suitable marker, sedimenting only slightly behind RSV DNA polymerase (Figure 8B). Thus, in gradients of 10-30% glycerol containing .2M KCl, the RSV DNA-dependent DNA polymerase has an S value of 7.1, corresponding to a molecular weight of ca. 95,000 Daltons.

Extent of purification by means of rate-zonal sedimentation. ^{3}H amino acid labeled virus was disrupted with non-ionic detergent and analyzed by rate-zonal sedimentation in glycerol gradients in order to determine the percent of the ^{3}H amino acids migrating into the 7S region, a value which would provide an upper limit for the amount of RSV protein comprising DNA polymerase peptides. As can be seen in Figure 9, none of the ^{3}H amino acid radioactivity migrated into the region of the gradient containing the enzyme, but rather, sedimented much more slowly (ca. 2-3S). When the peak fractions of the ^{3}H amino acids were recovered from the glycerol gradient and analyzed by polyacrylamide gel electrophoresis, the major virus-specific peptides were clearly evident (Figure 10A). A typical gel pattern of RSV

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Figure 8. Glycerol gradient centrifugation of DNA-dependent DNA polymerase with marker enzymes.

Panel A--40μl purified RSV (48μg protein) was disrupted with 1.0% NP_{40} for 30 minutes at 4° after which the volume was increased to 0.2 ml by the addition of 0.1M NaCl-0.002M EDTA-0.02M Tris-HCl, pH 8.1-0.2% (v/v) β-mercaptoethanol. 20μl of E. coli DNA-dependent RNA polymerase purified by the method of Burgess (1969) and provided by Dr. H. Eisen, was added and the sample was layered on a preformed 11.8 ml gradient of 10 to 30% glycerol in 0.05M Tris-HCl, pH 8.1-0.2M KCl-0.2% (v/v) β-mercaptoethanol, and centrifugation was conducted in an SW-41 rotor at 40,000 r.p.m. for 20 hours at 4°. After centrifugation, 0.48 ml fractions were collected; 50μl samples from each fraction were assayed in 100μl of the cocktail of Burgess (1969), lacking bovine serum albumin and containing 12.5 M ^{3}H GTP (0.8 Ci/mM), for 15 minutes at 37°. RSV DNA-dependent DNA polymerase was assayed by adding salts, substrates, and DNA to 100μl samples of each fraction to give a final volume of 125μl. ^{3}H TTP was added to .25μM (0.3X) and incubation was for 2 hours at 37°. ^{3}H GMP incorporation (○—○—○); ^{3}H TMP incorporation (●---●---●).

Panel B--40μl of purified RSV was disrupted and diluted as above. 5μl of E. coli alkaline phosphatase (Worthington) was added to a gradient prepared as above and centrifuged at 40,000 r.p.m. at 4° for 24 hours. DNA polymerase activity was assayed as above (○——○). Alkaline phosphatase was assayed by placing 100μl fraction samples in 3.0 ml of 1mM p-nitrophenylphosphate in 1.0M Tris, pH 8.1, and measuring the increase in optical density at 410 nm in 5 min.(●---●)

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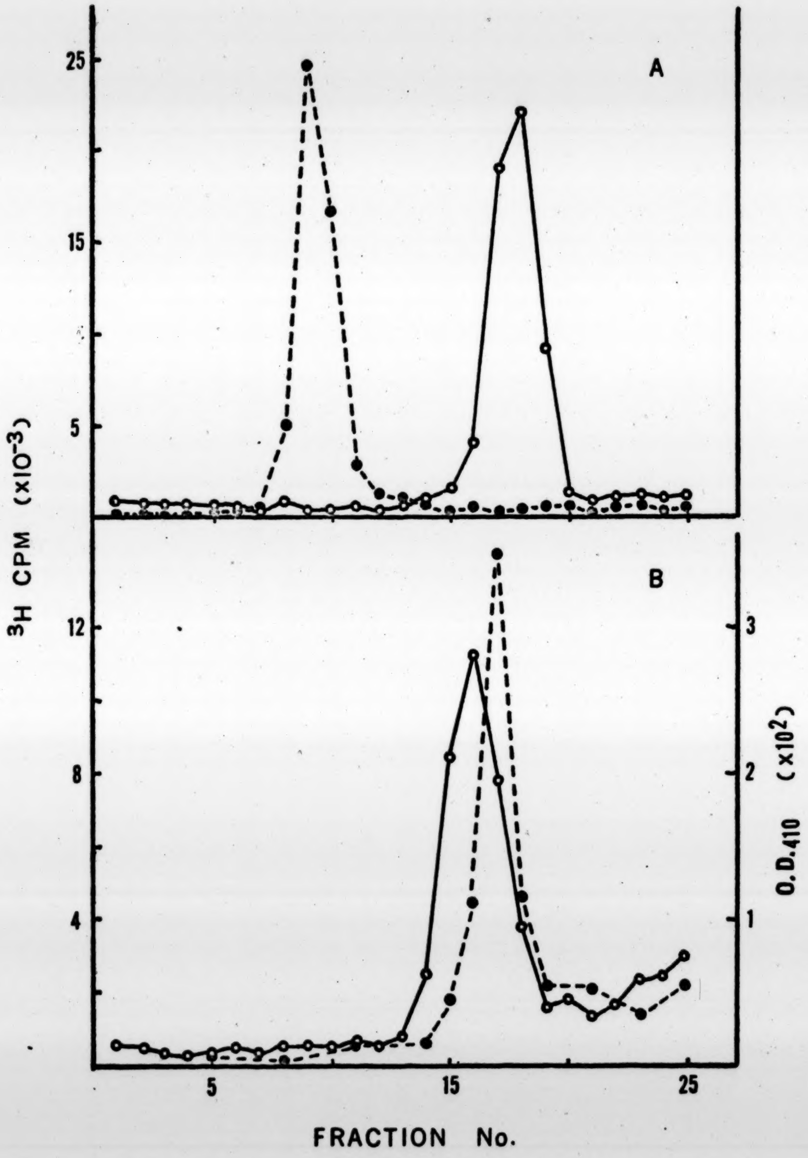
A
B
^{3}H CPM (x10^{-3})
25
15
5
12
8
4
O.D.410 (x10^2)
3
2
1
5
15
25
FRACTION No.

Figure 9. Relative rates of sedimentation of RSV peptides and DNA-dependent DNA polymerase in glycerol gradients. 0.3 ml of purified RSV containing 300 μg of RSV protein and 80,000 acid-precipitable ^{3}H amino acid c.p.m. was dialyzed against 0.1M NaCl-0.002M EDTA-0.02M Tris-HCl, pH 7.4, and disrupted for 30 minutes at 0° in 1.0% (v/v) NP_{40}, and layered on a glycerol gradient like that described in Figure 8. Centrifugation was at 40,000 r.p.m. at 4° for 43 hours. 10 μl samples from the collected fractions were added to 100 μl of a standard reaction mixture containing 200 μg/ml calf thymus DNA, 0.08mM unlabeled TTP, no unlabeled dGTP, and α-^{32}P-labeled dGTP, and DNA polymerase activity was assayed by incorporation of ^{32}P dGTP into acid precipitable c.p.m. in a 2 hour assay at 37° (○—○—○). One-half of the remaining volume of fractions 33, 34 and 35 and the remainder of each of the other fractions was acid-precipitated for the determination of the distribution of RSV ^{3}H amino acids (●---●---●). The values for fractions 33-35 were corrected for the volumes removed. The remainder of these fractions was used for the experiment described in Figure 10. Approximately 25% of the 80,000 ^{3}H amino acid c.p.m. were recovered from the gradient.

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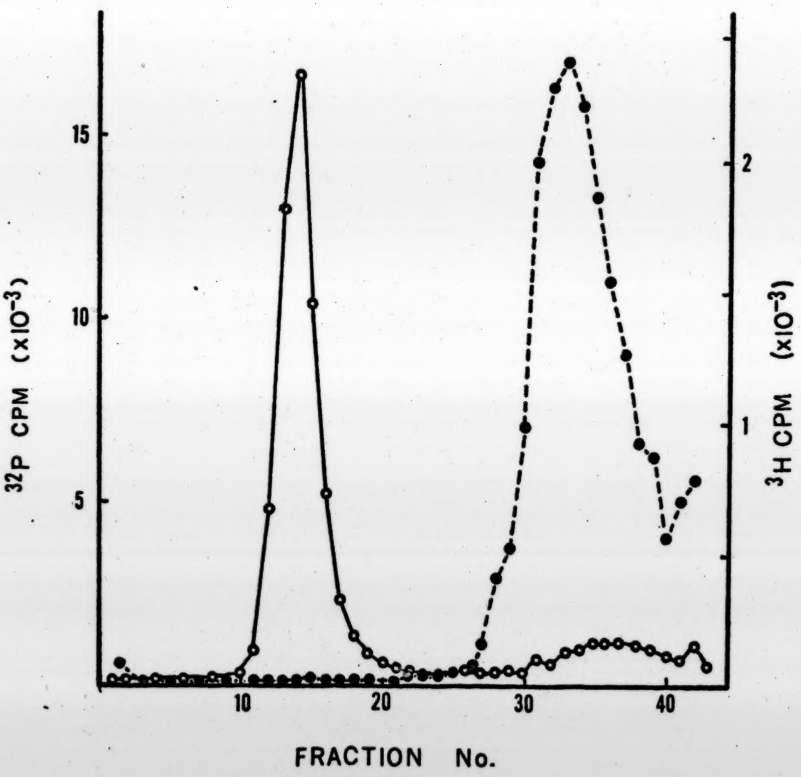

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Figure 10. SDS-polyacrylamide gel electrophoresis of ^{3}H amino acid-labeled RSV proteins obtained from a glycerol gradient.

Panel A--The peak fractions of ^{3}H amino acid-labeled RSV, obtained as described in Figure 9, were pooled and dialyzed overnight against 0.01M $NaPO_4$ buffer, pH 7.2. 0.25 volumes of a solution containing 5% SDS and 5% β-mercaptoethanol in 0.05M $NaPO_4$ buffer, pH 7.2 was added, and the sample was incubated at 100^o for one minute. The sample was dialyzed against 0.1% SDS in 0.01M $NaPO_4$ buffer, pH 7.2 for 4 hours, and then mixed with 1 μl of a bromephenol blue solution and made ca. 10% with respect to sucrose. This sample was layered on a 13.5% polyacrylamide gel and electrophoresis was conducted at ambient temperature at 6.5mV/gel until the bromephenol blue indicator had migrated 7.5 centimeters. The gel was sliced into 1 mm sections and counted. ^{3}H amino acid c.p.m. (●—●—●).

Panel B--Electrophoretic pattern of purified ^{3}H amino acid-labeled RSV obtained by Dr. J. Taylor in this laboratory (●—●—●).

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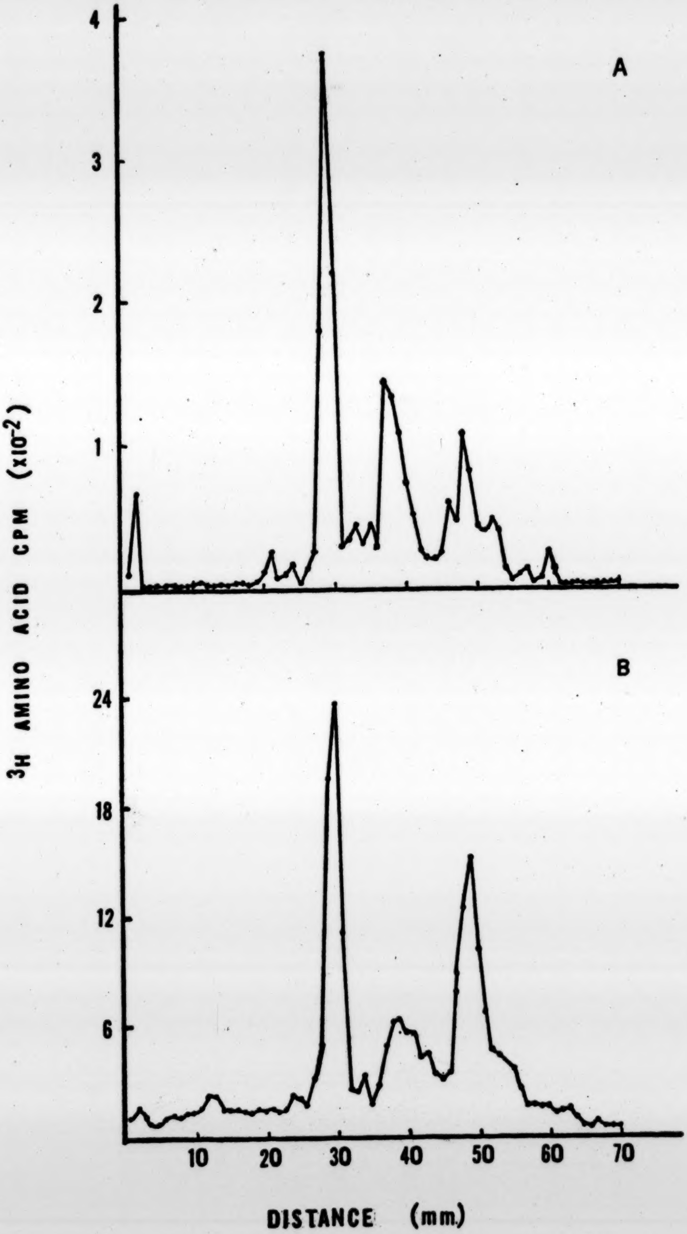
A
B
^{3}H AMINO ACID CPM (x10^{-2})
4
3
2
1
24
18
12
6
10
20
30
40
50
60
70
DISTANCE (mm)

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peptides is represented in Panel B of Figure 10 for comparison.

Rate-zonal sedimentation analysis of ^{3}H-fucose labeled virus was conducted to ascertain the sedimentation properties of the virus-specific glycopeptides with respect to the DNA polymerase (Figure 11). The great majority of the labeled glycopeptides sediment distinctly more rapidly than the enzyme with only the trailing edge of the ^{3}H fucose profile overlapping the DNA polymerase region. In these conditions of sedimentation neither the major virus-specific peptides nor glycopeptides co-sedimented with the enzymatic activity.

Catalytic properties of the isolated DNA-dependent DNA polymerase. The enzyme obtained from gradients of glycerol was occasionally obtained in 100% yields and is maximally active in the same conditions of assay as the virion-associated activity, except that there is no longer a requirement for non-ionic detergent. The isolated enzyme is also as stable as its virion-associated counterpart in the conditions of assay, displaying linear kinetics of ^{3}H TMP incorporation for 6 hours (Figure 12A). The virion-associated and isolated DNA polymerases also respond similarly to varying concentrations of calf thymus DNA (data not shown) and to DNA from other sources (Table II). Likewise, the monovalent cation inactivation curves for the two activities are similar (Figure 12B). All indications are then, that the isolated DNA-dependent polymerase possesses the same catalytic features as the virion-associated DNA-dependent polymerase, a consequence which argues against any virion-specific steric requirement for activity of this enzyme.

Figure 11. Relative rates of sedimentation of RSV glycopeptides and DNA-dependent DNA polymerase in glycerol gradients. 0.5 ml of purified RSV containing 160,000 c.p.m. ^{3}H fucose-labeled glycopeptides was dialyzed and disrupted as in Figure 9. The samples were layered on gradients of glycerol prepared as in Figure 9, and centrifuged in an SW-41 rotor at 40,000 r.p.m. for 37 hours at 4°. DNA-dependent DNA polymerase activity was assayed as in Figure 9 (○——○). The remainder of the gradient fractions were processed for acid-precipitable ^{3}H fucose (●- - - - -●). 110,000 c.p.m. ^{3}H fucose was recovered from the gradient fractions and 15,000 c.p.m. were recovered from the pellet.

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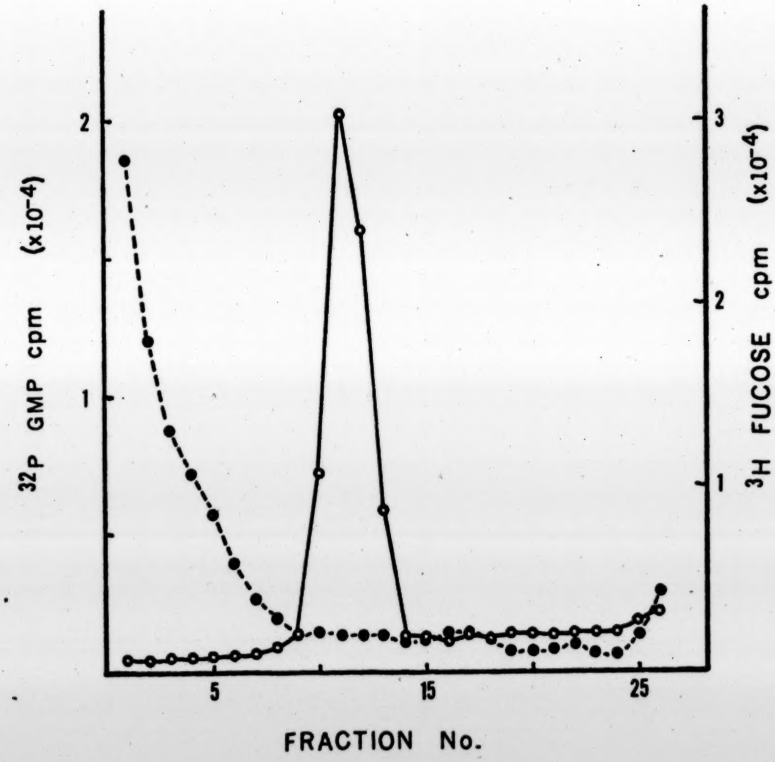

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Figure 12. Properties of the isolated DNA-dependent DNA polymerase. 0.5 ml of purified RSV (0.8 mg RSV protein) was fractionated in 31% ammonium sulfate as described in Table I and resuspended in 0.3 ml of 0.1M NaCl-0.002M EDTA-0.02M Tris-HCl, pH 7.4. The sample was layered on a density gradient of glycerol and centrifuged as described in Figure 9 for 38 hours. 10 µl samples of the fractions collected from this gradient were assayed for DNA-dependent DNA polymerase in standard reactions of 100 µl. The peak fraction (18,000 c.p.m. in a 2 hour assay) was utilized as the source of isolated DNA-dependent DNA polymerase in these experiments.

Panel A--Kinetics of ^{3}H TMP incorporation by virion-associated and isolated DNA-dependent DNA polymerase. 100 µl of isolated DNA polymerase was incubated at 37° in a standard reaction (1.0 ml) containing calf thymus DNA at 200 µg/ml, and 100 µl withdrawals for determination of acid-precipitable radioactivity were taken at the indicated times. 25 µl of purified RSV (15 µg RSV protein) was incubated in an 0.25 ml standard reaction containing 0.1% NP$_{40}$ and 200 µg/ml calf thymus DNA. 20 µl withdrawals were taken at the indicated times. Virion-associated DNA-dependent DNA polymerase activity(○———○); Isolated DNA-dependent DNA polymerase activity(●- - - - -●).

Panel B--Relative inactivation of virion-associated and isolated DNA-dependent DNA polymerase activities by increasing concentrations of NaCl. Reaction mixtures identical to those described in Panel A for the isolated DNA-dependent DNA polymerase (50 µl), and for the virion-associated DNA polymerase(50 µl) were incubated for 2 hours at 37° in the indicated concentrations of NaCl. Symbols are as in Panel A.

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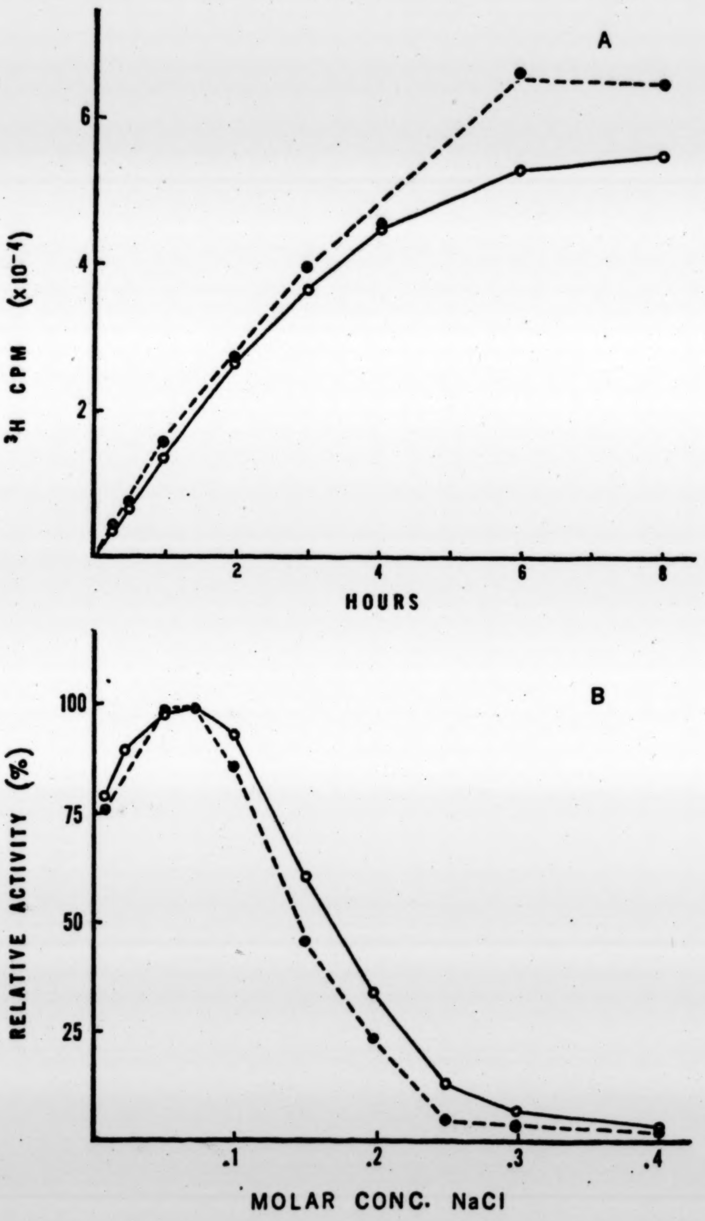
A
6
4
2
^{3}H CPM (x10^{-4})
2
4
6
8
HOURS
B
100
75
50
25
RELATIVE ACTIVITY (%)
.1
.2
.3
.4
MOLAR CONC. NaCl

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Table II
Template specificity of virion-associated and isolated DNA polymerase

Template (40 µg/ml)	RSV-associated DNA polymerase	Isolated DNA polymerase
	Relative activity	
Calf thymus DNA	1.0	1.0
Denatured calf thymus DNA	1.0	--
DNA	.05	.06
Dialyzed λ DNA	--	.06
Sonicated λ DNA	--	.11
Sonicated chick embryo fibroblast DNA	.30	.32

Standard 2 hour assays were carried out using either detergent disrupted purified RSV or gradient isolated DNA polymerase, and DNA at 40 µg/ml from several sources as template. Calf thymus DNA phage λ DNA and chick embryo fibroblast DNA were sonicated as described in Materials and Methods. Dialyzed phage λ DNA was dialyzed against 0.1M Tris-HCl, pH 8.1 for 72 hours.

Template specificity of the isolated DNA-dependent DNA polymerase. Several synthetic polynucleotides have been shown to be capable of stimulating incorporation of deoxyribonucleotides by the DNA polymerases of the RNA tumor viruses (Spiegelman et al., 1970; Scholnik et al., 1970). Moreover, Baltimore and Smoler (1971) have shown that in contrast to *E. Coli* DNA polymerase I, the AMV DNA-polymerase shows a clear preference for ribo- and deoxyribo- hybrid polymers over double-stranded deoxyribopolymers as templates for DNA synthesis. The assay conditions necessary for rA:rU and rA:T to function as templates for the RSV DNA polymerases have been developed in this laboratory by Dr. J. Taylor, who demonstrated that the isolated DNA polymerase responds to these synthetic templates in a fashion similar to that of detergent-disrupted virions. This result lent credence to the possibility that the isolated DNA-dependent DNA polymerase may also possess the active site for the RNA-dependent activity. To test for this possibility, it was deemed advantageous to isolate the enzyme in gradients containing low levels of monovalent cations because of the extreme sensitivity of the endogenous RNA-dependent activity to these salts (see Figure 1C). While these conditions resulted in an enzyme peak free of endogenous activity, the distribution of the activity in the gradient was over a much wider region, indicating that aggregation effects occur in the low salt gradient buffers (Figure 13A). When fractions of this gradient were supplemented with RSV-RNA or transfer RNA, the former gave an activity profile similar to that of DNA, albeit with a much lower specific activity, while transfer RNA failed to stimulate incorporation (Figure 13B).

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Figure 13. Template specificity of RSV DNA polymerase sedimented in a low salt glycerol gradient. 60 μl purified RSV (72 μg RSV protein) was disrupted for one hour at 0° in 1.0% NP_{40}. The sample was then diluted to 0.3 ml with 0.002M EDTA in 0.02M Tris-HCl, pH 8.1 with 0.2% (v/v) β - mercaptoethanol. This sample was layered on a preformed 4.4 ml gradient of 10 to 30% glycerol in 1.0mM EDTA-0.02M Trsi-HCl, pH 8.1, and 0.2% β - mercaptoethanol with an 0.3 ml 100% glycerol cushion. Centifugation was in an SW-65 rotor at 64,000 r.p.m. for 4 hours at 4°. Fractions of 0.2 ml were collected and each was divided into four 50 μl aliquots.

Panel A--DNA-dependent activity was assayed by adding 50 μl of a cocktail containing 0.16mM unlabeled deoxynucleoside triphosphates, 0.02M $MgCl_2$, 400 μg/ml calf thymus DNA and 1.6 M ^{3}H TTP in 0.1M Tris-HCl, pH 8.1. Incubation was for 2 hours at 37°. DNA-dependent activity (○—○—○).

Panel B--Endogenous and RNA-dependent DNA polymerase activities were assayed by adding 50 μl of a cocktail containing 0.16 M ^{3}H TTP in 0.1mM unlabeled deoxynucleoside triphosphates, 0.02M $MgCl_2$, and 1.6 μM ^{3}H TTP in 0.1M Tris-HCl, pH 8.1 with either a) no additional nucleic acid for endogenous activity (●—●—●);
b) 2 μg/ml HeLa transfer RNA (○—○—○); or
c) 2 μg/ml RSV RNA (○- - -○- - -○).

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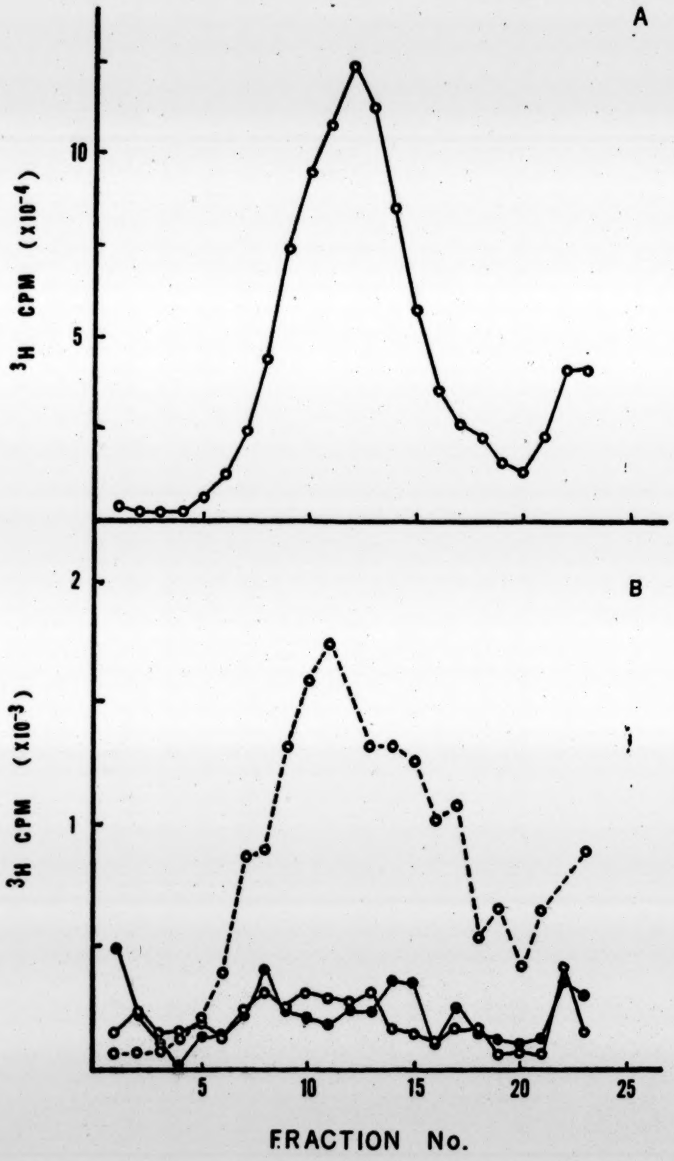

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Properties of the DNA synthetic reaction stimulated by RSV RNA. RSV RNA at a concentration of 2 μg/ml saturates the DNA polymerase (Table III). The kinetics of the reaction in which RSV RNA serves as template is compared with a like reaction in which a similar concentration of calf thymus DNA is employed to direct DNA synthesis in Figure 14. To answer the critical question of whether the RNA is being faithfully transcribed into DNA of complementary base composition, the nucleic acids of an RNA-directed reaction similar to that of Figure 14 were extracted and analyzed by rate-zonal sedimentation. A portion of the product RNA was found to migrate into the 70S region of the gradient, a result which at once suggested that the input RNA is not being degraded, and that this DNA product is complementary to and hydrogen-bonded with the template RNA. This experiment is analogous to those originally used to identify the early product of the DNA polymerase reactions of RSV virions (Garapin et al., 1970) (Figure 15A). After ribonuclease digestion, the 70S DNA is displaced into the 4S region of the gradient, suggesting that this DNA, like that of the virion reaction, is of rather small size (Figure 15B). Other sources of RNA were also tested for their ability to stimulate DNA synthesis. Of these sources, only poliovirus RNA was found to serve as template for the synthesis of DNA, and it was active to a lesser extent than RSV RNA (Table IV).

Chromatography of the RSV-DNA polymerase. Attempts to attach the DNA polymerase to and elute it from DEAE cellulose or phosphocellulose proved negative in that all of the activity

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Table III
RSV RNA saturation curve

Concentration of RSV RNA	Counts/Minute	Relative Activity
0	230	.12
0.1 μg/ml	450	.24
0.4 μg/ml	820	.44
2.0 μg/ml	1850	1.00
4.0 μg/ml	1700	.92
8.0 μg/ml	1525	.82

Samples of 10 μl from fraction #7 of the glycerol gradient described in Figure 14 were assayed in 50 μl reactions containing 4.0 μM ^{3}H TTP and RSV RNA at the indicated concentrations for 1 hour at 37°. Acid-precipitable ^{3}H TMP incorporation was determined.

Figure 14. Kinetics of DNA synthesis directed by RSV RNA. The DNA polymerase for this experiment was prepared by first fractionating 0.54 ml of purified RSV (1.0 mg RSV protein) by ammonium sulfate precipitation (Table I), and redissolving the precipitate in 0.3 ml of 0.002M EDTA-0.2%β-mercaptoethanol-0.02M Tris-HCl, pH 8.1. Sedimentation of this sample was similar to that described in Figure 13 except that centrifugation was for 6 hours. DNA-dependent activity was distributed similarly to Figure 13A, and the activity peak was at fraction 8, with 40,000 c.p.m./10μl in a 2 hour assay. No endogenous activity was detected. DNA polymerase activity responsive to calf thymus DNA was assayed by adding 20μl of the peak gradient fraction to 80μl of a standard reaction mixture containing DNA at 2μg/ml and 4.0μM ^{3}H TTP (5X). 10μl withdrawals were taken at the indicated times for the determination of acid-precipitable radioactivity. DNA polymerase activity stimulated by RSV RNA was assayed in the same fashion as above except that calf thymus DNA was replaced by RSV RNA at 2μg/ml.

Calf thymus DNA at 2.0μg/ml (●---●---●).

RSV RNA at 2.0μg/ml (○——○——○).

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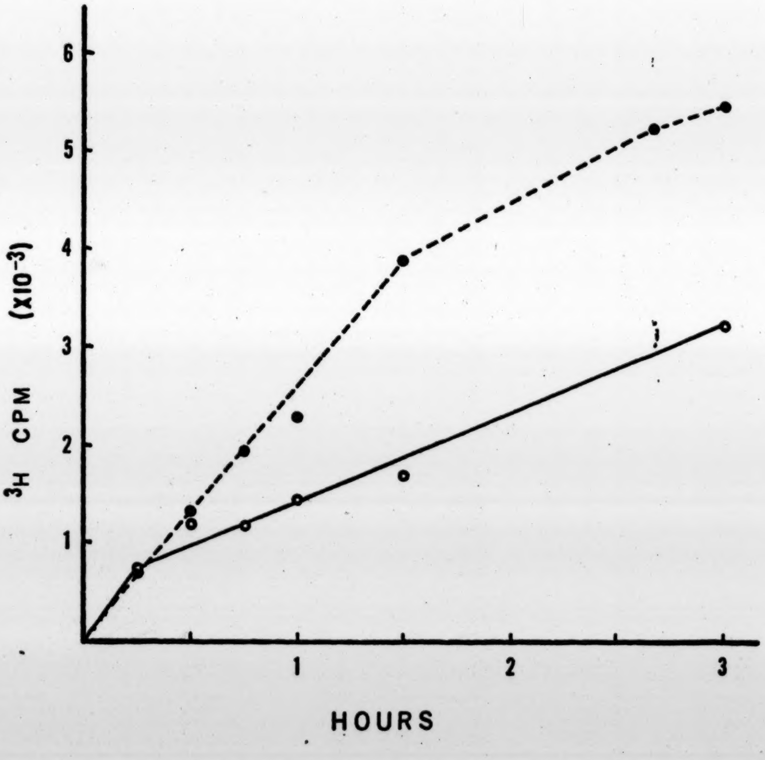

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Figure 15. Rate-zonal sedimentation of product synthesized in response to RSV RNA. An 0.1 ml reaction, identical to that described in Figure 14, with RSV RNA present at 2.0 m/ml was incubated for 3 hours at 37° after which 5μl 10% (w/v) SDS and 5μl of 10 mg/ml self-digested pronase were added, and further incubation was conducted for 45 minutes at 37° to dissociate the nucleic acids from protein.

Panel A--A 50μl portion of this sample containing 3500 acid-precipitable c.p.m. was mixed with ^{32}P-labeled RSV 70S RNA and layered on a preformed 5.0 ml density gradient of 15 to 30% sucrose (w/v) in 0.1M NaCl-0.001M EDTA-0.02M Tris-HCl, pH 7.4. Centrifugation was conducted in an SW-65 rotor at 64,000 r.p.m. for 70 minutes at 4°. Fractions of ca. 0.2 ml were collected and processed for ^{3}H and ^{32}P acid-precipitable radioactivity. ^{3}H radioactivity (○—○—○). The arrow indicates the radioactivity peak fraction of the ^{32}P-labeled 70S RSV RNA.

Panel B--A second 50μl portion of the extracted nucleic acids was diluted to 0.2 ml with 3.0M EDTA, boiled pancreatic RNase was added to 10μg/ml and the contents were incubated at 37° for 15 minutes. The sample was then layered on a sucrose density gradient and processed as in Panel A. Acid-precipitable ^{3}H radioactivity (○—○—○).

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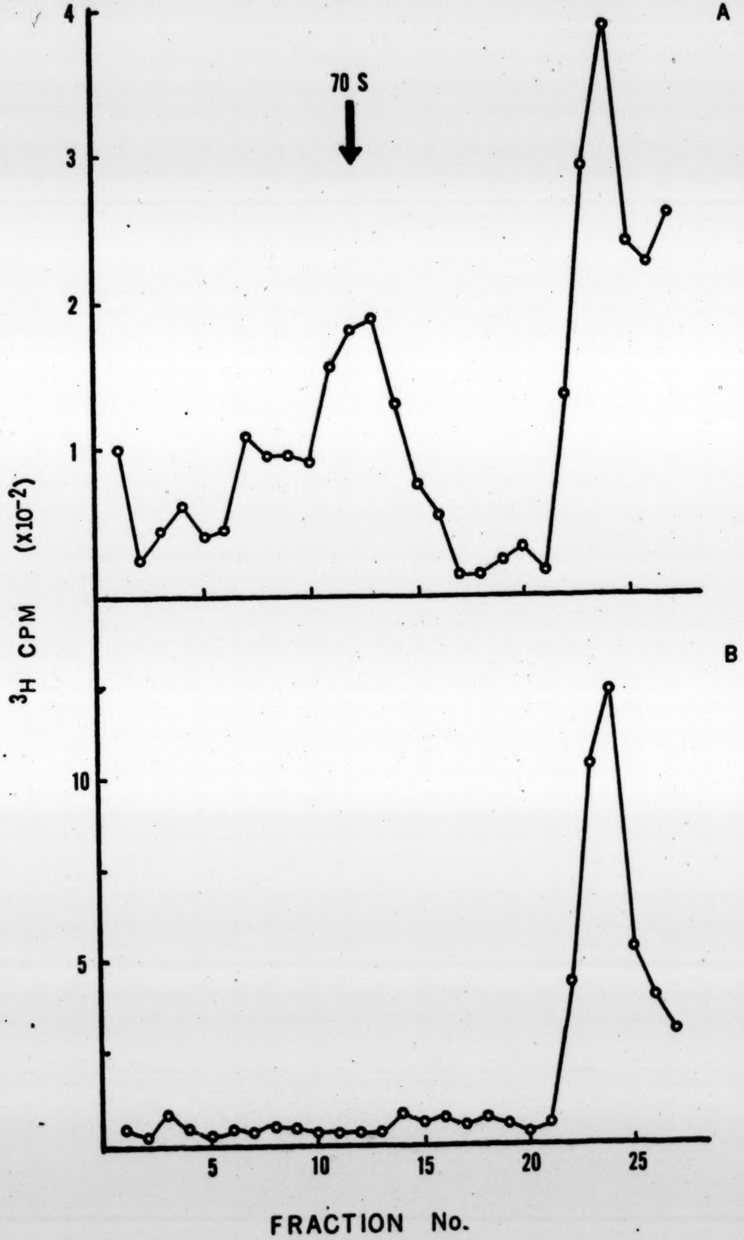

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Table IV
Response of isolated RSV DNA polymerase
to homologous and heterologous RNA

Template Nucleic Acid	Concentration	Activity in Peak Fraction	Activity Relative to RSV RNA.
RSV RNA	2 µg/ml	2030	1.00
HeLa tRNA	2 µg/ml	70	.03
HeLa Ribosomal RNA	2 µg/ml	140	.07
Poliovirus RNA	2 µg/ml	1070	.53
No Addition	2 µg/ml	160	.08
Calf thymus DNA	2 µg/ml	10,000	5.00
Calf thymus DNA	200 µg/ml	52,000	25.00

Samples of 10 µl from alternate fractions across the peak of the glycerol gradient described in Figure 14 were assayed for 1 hour at 37° in 50 µl standard reactions containing 4.0 µM ^{3}H TTP (5X), and RNA from the indicated sources at 2 µg/ml or calf thymus DNA at 2 and 200 µg/ml. Total acid-precipitable counts per minute from the peak fraction (fraction #8) for each added nucleic acid are indicated in the third column. The activity on either side of the peak for the RSV and poliovirus RNA's, as well as for the two concentrations of DNA, was distributed in a symmetrical fashion. The endogenous activity and that in the presence of either tRNA or ribosomal RNA was greatest at the bottom of the gradient (ca. 400 c.p.m.), gradually decreasing to less than 50 c.p.m. at the top of the gradient.

was lost. More recently, Dr. J.M. Bishop has been successful in utilizing both DEAE cellulose and phosphocellulose for chromatography of the enzyme (unpublished results). In each case, success has been coincedent with the addition of non-ionic detergent to the gradient buffers (NP_{40} or triton X).

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Discussion

The RNA tumor viruses have long been looked upon with great interest because of the insights they might provide into the process of oncogenesis. These viruses however, have until recently remained refractory to all attempts to trace the molecular events of their intracellular growth cycles, because virus-specific RNA, be it complementary (-) stranded, ribonuclease resistant double-stranded or polysomal messenger, had never been convincingly detected. This state of affairs was attributed on the one hand to the low rates of virus-specific RNA synthesis, and on the other to the fact that the utilization of inhibitors for the prupose of reducing the background of host-specific RNA or DNA synthesis also invariably block virus-specific synthesis as well. As a consequence, biochemical studies of the oncogenic RNA viruses were pretty much limited to characterization of the macromolecular contents of the virion and to the effects of inhibitors on infection, cellular transformation, and progeny virus synthesis (for review see Green, 1970). For purposes of perspective it might be noted that perhaps the most definitive of these studies was that of Duesberg and Vogt (1969) wherein it was demonstrated that infection by one type of RNA tumor virus does not subsequently relieve the requirement for DNA synthesis in the establishment of infection by a superinfecting virus. The results of this study, in conjunction with the inhibitory effect on progeny virus synthesis at all times by actinomycin D, were probably the most decisive arguments favoring the hypothesis that the tumor virus genome must be

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transcribed from RNA into DNA, and thence from DNA to RNA, as essential steps in the virus growth cycle. Such evidence, however, was hardly sufficient to persuade the preponderance of tumor virologists to abandon their conventional concepts of the flow of genetic information in searching for a solution to the enigma of the mode of replication of the RNA tumor viruses.

The discovery of DNA polymerase activities in RSV and Rauscher mouse leukemia virus then, immediately swung wide the gate to new avenues of investigation into the modes of replication of and oncogenesis by these viruses. It should be pointed out, however, that to date no conclusive experimental evidence has been forthcoming which has confirmed that DNA serves as an intermediate in the replication of the genomes of the RNA tumor viruses. There is now good evidence that all RNA tumor viruses possess DNA polymerase activities (Schlom et al., 1971). There is also good evidence that the entire genome of RSV is transcribed *in vitro* into DNA of complementary base composition (Duesberg and Canaani, 1970), and that at least a sizable portion of the genome can be transcribed into double-stranded DNA *in vitro* (Varmus et al., 1971). But, perhaps the most persuasive evidence that this enzymatic step is biologically significant has come from the laboratory of Hanafusa and Hanafusa (1971). They have demonstrated that preparations of RSV $\alpha(0)$, a non-infectious, helper-free variant of RSV (0), lacks detectable levels of endogenous RNA-dependent or exogenous DNA-dependent polymerase activities. When cells from which the DNA polymerase deficient RSV $\alpha(0)$ is obtained are superinfected with an avian leukosis virus, the resulting ALV (and presumably the

XUM

phenotypic RSVα(ALV) as well), possesses the DNA polymerase activities. This adds up to a potent but inconclusive argument that the virion polymerases are not simply host cell enzymes that have been incidently incorporated into the virions, because virus genotypes alternately lacking and possessing the DNA polymerase activities can be obtained from the same cells. Decisive evidence, however, such as the demonstration of complete DNA complements of the RSV RNA within infected or transformed cells or the synthesis or isolation of infectious and/or oncogenic DNA, remains to be put forward.

This uncertain state of affairs should not, however, detract from the real as well as potential importance of these polymerases in the investigation of the biology of the RNA tumor viruses. It is clear that there are present within the RSV virion both endogenous RNA-dependent and exogenous DNA-dependent DNA polymerases which require the presence of non-ionic detergent for activation, but which otherwise respond to rather typical ionic and substrate conditions (Figures 1,3,4,5 and 12). It is also clear that unlike previously characterized virion-associated polymerases, these activities can be easily and completely separated from any association with nucleoprotein core structures, and can thus be rendered totally dependent on exogenous nucleic acid for activity (Figures 6,7,8,9,11 and 13).

Studies with amino acid labeled and fucose labeled viruses also strongly suggest that the peptide(s) of DNA-dependent activity cannot be attributed to any of the major peptide or glycopeptide components of the virion(Figures 9 and 11). On the basis of the experiment described in Figure 9, where none of

XUM

the 20,000 c.p.m. detected in the gradient were within the polymerase region, a value of less than 0.5% is arrived at for the upper limit of the percent of RSV protein comprised by DNA-dependent DNA polymerase peptides. A lower value was not assigned because fewer than 100 c.p.m. of ^{3}H amino acids in the enzyme protein would not likely be within the limit of detection by this technique. This low enzyme versus virion protein ratio makes it technically difficult to obtain sufficient labeled or stainable enzyme protein to characterize the subunit size and composition by means of SDS-gel electrophoresis. Although much less reliable than sizing on SDS gels, the active enzyme can be assigned a molecular weight of approximately 95,000 Daltons from the sedimentation coefficient obtained by rate-zonal sedimentation of the activity with marker enzymes in gradients of glycerol (Figure 8).

The template specificity of the DNA-directed activity has not been precisely delineated in these studies, although it would appear that DNA from calf thymus serves better in this capacity than other sources of DNA (Table II). While denatured calf thymus DNA apparently serves equally well in stimulating DNA synthesis, it can by no means be ruled out that short, preexisting, renatured, or hairpin double-stranded regions are serving to initiate this synthesis (Figure 5B). Also, the poor response of the enzyme to intact phage-λ DNA suggests that either specific initiation sequences are required or that virtually completely double-stranded DNA molecules are poor templates. It must be noted that none of these observations could be used as an argument against the proposal that the RSV DNA polymerases

XUM

have template requirements similar to enzymes involved in the repair of DNA, as suggested by Hurwitz et al. (1971).

By all criteria tested, the DNA-dependent enzyme isolated by means of rate-zonal sedimentation in gradients of glycerol behaves identically to the virion-associated activity (Figure 12 and Table II). Actually, the isolated enzyme no longer requires non-ionic detergent for activation, but the virion enzyme presumably requires detergent treatment only for purposes of bringing about dissolution of the virus envelope, a requirement which, of course is not a factor in eliciting the isolated activity. The most important aspect of the rate-zonal sedimentation purification step, however, is that in addition to freeing the DNA-dependent activity of any endogenous RNA-dependent activity, the sedimentation coefficient of this protein is sufficiently great to sediment it completely free of ribonucleases known to be present in purified RSV preparations (Quintrell et al., 1971). The conclusions that the enzyme preparation is free of ribonuclease is arrived at by the demonstration that DNA synthesized in vitro can be extracted from a reaction after 3 hours of incubation which sediments in the 70S region prior to ribonuclease digestion (Figure 15A), and at ca. 4S after RNase digestion (Figure 15B). This finding is analogous to those obtained from study of the endogenous RNA-dependent reaction (Garapin et al., 1970), and represents key evidence that the exogenous RNA is being faithfully transcribed into DNA of complementary base composition. Other laboratories have recently reported evidence of DNA synthesis dependent on tumor virus RNA (Duesberg et al., 1971; Green et al., 1971), but none have as yet demonstrated the specificity of the DNA product by this or other means. While all such experiments reported here must be considered preliminary in that they were conducted with a total of two enzyme preparations and have not been repeated with enzyme purified in this manner, it would appear that the protein component which synthesizes DNA using DNA as template also contains the catalytic site which utilizes RSV RNA, and to a lesser extent, poliovirus RNA as template for the synthesis of DNA (Figure 13 and Table III). Duesberg et al. (1971) have also arrived at a similar conclusion.

A second feature of the DNA synthesized by the isolated polymerase in response to RSV RNA is its small size (ca. 4S), as is demonstrated in Figure 15B. While it is difficult to envisage a biological role for DNA of this size, this state of affairs is likely to persist until and unless DNA comparable in size to the RNA genome of these tumor viruses is detected.

This brings up yet another obstacle presented by the oncogenic RNA viruses to attempts to penetrate their enigmatic ways. For though the genetic repository of these viruses would appear to be constituted by a 70-80S RNA molecule of from 9-12 million Daltons, Peter Duesberg (1968) has shown that when subjected to conditions which dissociate hydrogen-bonded molecules (Dimethyl sulphoxide and gentle heat) the 70-80S RNA is dissociated into 36S molecules of ca. 3 million Daltons, and possibly into smaller molecules as well. While Duesberg and Vogt (1970) speculated that several non-identical subunits, including a subunit responsible for cellular transformation, may make up the 70-80S genome, others have concluded that the subunits are identical, and thus, redundant (Bader and Steck, 1969). Moreover, there are present within purified RNA tumor virus preparations small quantities of 28S, 18S and 7S RNAs (Bonar et al., 1967; Bishop et al., 1970b) and rather large quantities of 4S RNA (Robinson and Baluda, 1965; Bishop et al., 1970a), which has been shown to be transfer RNA (Bonar et al., 1970; Erickson, 1969). Although the presence of most if not all of these smaller RNA components may be accounted for by the trivial explanation that they represent host cell RNA components contributed by cellular microsomes having the same rate-zonal and isopycnic sedimentation characteristics as the RNA tumor viruses, it is not so easy to write off as insignificant the 7S RNA, and the smaller RNA components from the 70-80S complexes. All of this is by way of pointing out the fact that it is inherently difficult to delineate biologically relevant virus-specific DNA, when the biologically relevant RNA of these viruses has not been properly defined.

It is frustrating that the functional roles of the virion-associated DNA polymerases have not been elucidated after a year of study. To justify the rate at which progress has been made, however, one need only consider that there was an eight year interim between the time that the *E. coli* DNA-dependent RNA polymerase was purified to homogeneity and the time that the complete system for biologically relevant RNA synthesis by this enzyme *in vitro* was defined (Roberts, 1969). It was a full decade before the complete system for controlled gene expression *in vitro* was delineated (De Crombrugghe et al., 1971). This line of reasoning can proceed from the sublime to the

XUM

ridiculous if only the status of in vitro replication (as opposed to repair) of DNA is contemplated.

Regardless of the biological significance of these RNA tumor virus-associated DNA polymerase activities, their utility has already been demonstrated in that the DNA synthesized by such enzymes hybridizes specifically with its homologous RNA, and hence can be used as a specific probe for the titration of the previously undetectable intracellular RSV RNA (Garapin et al., 1971; Green et al., 1971). While (+) strand RSV RNA may be present in quantities representing ca. 0.5% of cellular RNA, (-) strand RNA has not been detected in experiments sufficiently sensitive to detect such RNA if it were present in quantities equal to 1% of that of the (+) strand (J. Leong, 1971).

It might be noted that if such "reverse transcription" can be adapted so as to use heterologous messenger RNA species for the synthesis of DNA complments, it might have potential as a method for preparing DNA for the purpose of titrating specific messenger RNA species in eucaryotic systems. Support for the feasibility of this prospect comes from the propensity of this enzyme to utilize as template for DNA synthesis a variety of ribo- and deoxyribopolymers (Spiegelman, et al., 1970), and its response to poliovirus RNA (Table IV). These observations suggest the likelihood that this enzyme does not possess the strict template requirement displayed by the phage Qβ replicase. Further support for a more general RNA template requirement has recently come from Dr. Duesberg's laboratory. It has been shown that while dissociated 70S RSV RNA does not serve as template, DNA synthesis at a rate higher than with native 70S

XUM

RNA occurs with both native and denatured 70S RNA if oligodeoxythymidylic acid is also present (Duesberg et al.,1971). It may well be that double-stranded regions rather than specific base sequences represent the primary factor in the propensity for a nucleic acid to serve as template for this enzyme.

At the time this is being written--approximately one year after the discovery of virion-associated DNA polymerase activity in RNA tumor viruses--nothing is yet certain regarding the intracellular modes of replication and oncogenesis by these viruses. It is clear, however, that concepts such as the "oncogene" theory of Huebner and Todaro (1969) and the "provirus" and "protovirus" theories of Temin (1964a and 1970), are being examined much more patiently. It is also clear that erudition and dogma have given way to lively experimentation and serious consideration of unorthodox hypotheses. In this spirit, I would like to consider such a departure from the established concepts of information transmission and expression. If, as proposed by the "protovirus" and "oncogene" theories, the genetic information of the RNA tumor viruses resides not only in infected and transformed cells but also in uninfected cells, might it not also be possible that a self-perpetuating transmissible agent could exist which would not require the inclusion of genetic information in its transmissible form? Such an agent might only require the means to be transmitted to and incorporated into the host or target cell, after which it could serve as an effector for the expression of host genetic information, which would result in the synthesis of new effector molecules and the structural proteins necessary for their encapsulation preparatory to further transmission. Such an effector could be analogous to bacterial sigma factors (Burgess et al., 1969), and it could qualify as an infectious disease-eliciting agent if it concomitantly promoted the expression of genes deleterious to the host cell. The reason for proposing such an infectious agent, if one be necessary, is simply to make the point that in pondering the molecular events of cellular transformation brought about by the RNA tumor viruses, consideration of a possible role for virion-encapsulated protein effector may be justified. In this instance the effector might "turn on" genes for its own synthesis and those genes whose products would bring about cellular transformation. Meanwhile, the viral RNA would be independently replicated either through a DNA or RNA intermediate, and in the process provide the structural molecules for progeny virions. A further extention of this model would eliminate the need for the transmission of any genetic information what-so-ever. By this argument, then, it may not be axiomatic that informational macromolecules be ascribed to such enigmatic self-propagating agents as that for the disease scrapie and possibly Australia antigen. This hypothetical model for an "infectious sigma factor" may be at least as tenable as those calling upon "informational carbohydrates" or other such non-nucleic acid informational macromolecules to account for the missing nucleic acids of the "slow viruses" and Australia antigen.

Finally it might be said of the RNA viruses, which were considered to be biological if not medical oddities in the early 1960's, that they and their bacteriophage counterparts have been constantly yielding of information concerning the replication of nucleic acids and the means of expressing genetic information in RNA. Much of this information has been vital to the development and confirmation of many of the concepts of molecular biology. The dazzling biological complexity of the most simple of biological entities, the RNA bacteriophages, has been elucidated to a great extent, while the remarkable structural simplicity of these bacteriophages may conceivably result in the elucidation of complete three-dimensional molecular structure of one of them in the foreseeable future. Although double-stranded RNA and RNA-dependent RNA polymerases were originally discovered in eucaryotic systems, it has been the RNA bacteriophages which have been the most fertile area of inquiry. There is now re-awakening interest in the RNA viruses.

The picornaviruses have provided clues concerning contrasting mechanisms of translation of messenger RNA in procaryotic and eucaryotic organisms. And, now the large RNA viruses are presenting examples of widely variant mechanisms for the transfer and expression of information contained in RNA which could well have ramifications in progress towards the understanding of oncogenesis at the molecular level. The RNA viruses and bacteriophages have been and will continue to be vital to the elucidation of biological processes at the molecular level. They sure are fascinating little critters!

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